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THE TUMOUR VIRUS DISSEMINATED FROM FILTERABLE TO NON-FILTERABLE TUMOURS.

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It was recently shown that under certain circumstances the virus of the Rous No. 1 sarcoma can remain latent in the tissues of the host without exerting any neoplastic action (Carr, 1943*b*). This latent state recalls the experiments of Mellanby (1938), who demonstrated that when a Rous No. 1 and a non-filterable tumour are growing in the same host, the virus of the filterable tumour can often be detected in cell-free extracts of the non-filterable tumour, though the presence of the virus is not indicated by any alterations in the growth-rate or histological and cytological characteristics of the infected tumour. This transfer of virus to a non-filterable tumour was also found by Gye and Schlesinger (unpublished), who found that the virus of the MH/2 endothelioma would pass to a chemically-induced fibrosarcoma in the same host, the identity of the virus being indicated by the characteristic MH/2 histology of the tumour resulting from inoculation of the fibrosarcoma extract. Similarly, a Fujinami tumour passed a virus to non-filterable tumours which could be identified as Fujinami by its ability to infect ducklings. It is uncertain, however, whether such virus is in the ineffective " latent " condition in virus-sensitive cells, or whether it is merely the virus known to be constantly disseminated from filterable tumours into the blood and thus into the phagocytic cells of the host's organs, in which situations its neoplastic activity does not become manifest (Carr, 1944). If the virus content is due only to such disseminated virus, then only a small amount will be present; the actual quantity depends upon such factors as the volume of blood and number of phagocytic cells present in the tissue, but even in the richest known source, the spleen, it seldom exceeds 20 minimal infective doses (M.I.Ds.) per gramme of tissue (Carr, 1944). On the other hand, if many of the tumour cells are involved, a much greater content would be anticipated. Assuming a volume of $(10\mu)^3$ per cell, then if only 0·1 per cent of the cells were to contain a single associated tumour virus particle, about 10^6 particles per gramme would be present.

The experiments of Mellanby were not designed to indicate the actual concentration of tumour virus contained in the non-filterable tumours, but there was some suggestion that only a small amount was involved. In the present investigation, quantitative determinations of the contained virus were made, in the hope of deciding between the two possibilities.

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METHODS.

The experiments were carried out using Rous No. 1 sarcoma as the filterable tumour, and two non-filterable chemically-induced avian sarcomas, the GRCH/15 originated by Peacock and generously made available by him, and a methylcholanthrene-induced sarcoma Mca I originated at this Institute, and which it is hoped to describe in a subsequent publication. Grafts of either of the two non-filterable tumours were made into the breast muscles of chicks of the Institute flock, aged 6–9 weeks, and some time later the birds were inoculated with virus of the more rapidly-growing Rous No. 1 sarcoma in each leg. After a suitable time had elapsed the bird was killed, and a portion of each type of tumour was minced with scissors, extracted for some hours with 10 volumes of M/100 Na_2HPO_4 solution containing cyanide as oxidase inhibitor, and the extract clarified by centrifuging. Ten-fold dilutions of each extract were prepared, and the virus content determined by intramuscular inoculation of 0·5 ml. into groups of 3 young chicks.

RESULTS.

The results are summarized in Table I. It will be seen that the non-filterable tumour sometimes contained a small amount of the Rous No. 1 virus, but that none was found in most experiments. A negative finding indicated that in $3 \times 0{\cdot}5$ ml. of 10 per cent. tumour extract no virus was detected, i.e. there was

TABLE I.—*Virus Content of Filterable and Non-filterable Tumours Growing in the Same Host.*

Expt. No.	Rous tumour age (days).	Virus content M.I.Ds./g.	Non-filterable tumour.	Age (days).	Virus content M.I.Ds./g.
1	11	>200,000	GRCH/15	33	0
2	20	>20,000	,,	43	20
3	26	>20,000	,,	49	0
4	27	>20,000	,,	63	20
5	7	>200,000	Mca I	39	0
6	13	>200,000	,,	46	0
7	17	>200,000	,,	128	0

less than 1 M.I.D. in 0·15 g. of tumour. It seems that the small amount sometimes found can reasonably be attributed to the vascular portions of the tumour tissue rather than to the tumour cells themselves. In agreement with Mellanby's findings with other tumours, the contained virus did not affect the gross or microscopic appearance of the non-filterable tumour, which further suggests that it was not associated with the tumour cells.

It is possible to raise the objection that the tissue debris of the non-filterable tumour that is discarded after centrifuging had adsorbed a quantity of Rous No. 1 virus contained in the tumour. In order to control this possibility equal portions of filterable and non-filterable tumour taken from sites adjacent to the parts removed for extraction were minced together, extracted in the same way as the other tumours, and the activity compared with that of the extract of Rous No. 1 tumour alone. No appreciable decrease was noted in 2 experiments with GRCH/15 tumours, 2 experiments with Mca I tumours, or 2 similar experiments in which a GRCH/15 and an Mca I tumour were taken from hosts not growing a Rous tumour. Loss of virus by such adsorption can therefore be disregarded.

DISCUSSION.

It will be noted that the earlier growth of the non-filterable tumour in the host did not appear to reduce the virus content of the extract of the Rous No. 1 tumour later inoculated in the same host. It has been shown (Carr, 1943*a*) that a developing Rous No. 1 tumour will immunize the host against its contained virus in about 40 days. Consequently the above result suggests that neither the GRCH/15 nor the Mca I tumour contain an antigen which will immunize a fowl against the Rous No. 1 virus. This suggests that as far as the fowl is concerned, there is nothing in common between the Rous No. 1 virus and the two chemically-induced sarcomas employed. This is in contrast to the cross-neutralization observed by Gottschalk (1943) with the filterable sarcoma 13 and non-filterable sarcoma 16. Other workers have shown that cross-neutralizing sera can be obtained when the antibodies are produced in foreign species, but as similar relations can be shown between the virus and normal fowl tissues (Gye and Purdy, 1931), the significance of these findings is not easy to evaluate, and no attempt was made to carry out such investigations with the present material. This work does not indicate any attraction between the tumour virus and either the intact or damaged non-filterable tumour cell. Either such cells lack the specific receptors for the virus (suggesting that they are a different type of cell to that attacked by the Rous No. 1 virus), or the receptors are present but inactivated. A considerable amount of "heavy protein" are associated with both GRCH/15 and Mca I, and as Claude (1940) has shown that such material in many ways resembles the tumour viruses, the possibility that any receptors present are saturated with such "inactive virus" cannot be ignored.

SUMMARY.

Only small amounts of tumour virus are present in non-filterable GRCH/15 and Mca I tumours growing in the same host as a Rous No. 1 tumour, and are attributed to the virus disseminated about the vascular system of the host. The cells of neither of the non-filterable tumours will adsorb the tumour virus, nor do they contain an antigen producing antibodies to Rous No. 1 sarcoma virus in the fowl.

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THE EFFECT OF TREATMENT ON THE SPLEEN OF THE GOLDEN HAMSTER (*CRICETUS AURATUS*) INFECTED WITH *LEISHMANIA DONOVANI*.

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THE histopathology of kala-azar has been studied by a great many authors, and was accurately described in the early work of Christophers (1904). A good summary of the pathological anatomy of the disease in man and animals has been given by Laveran (1917). Shortt (1923) described the pathology of acute experimental kala-azar in monkeys, and Meleney (1925) made an extensive study of the histopathology in Chinese hamsters (*Cricetulus griseus*) infected with *L. donovani*, as well as in two human and two monkey cases. The lesions in the human cases which he studied were essentially the same as those found in the hamster. The histological and other changes which occur in the spleen of man or animals after treatment are practically unknown, although Adler and Tchernomoretz (1939) described them briefly in the case of hamsters infected with *L. donovani* after treatment with stilbamidine. The histological picture was, in their case, somewhat complicated by the fact that a number of laparotomies were performed on the same animal. In his chemotherapeutical studies of experimental leishmaniasis in golden hamsters, Goodwin (1945) considered the effects of treatment with antimonials in relation to the initial degree of infection, body weight and spleen size. He noted that the size of the latter organ was not proportional to the number of parasites present.

In the present investigation we have studied the changes in the spleen of the golden hamster infected with *L. donovani* during and after treatment. This animal was chosen because in it *L. donovani* causes a progressive fatal infection without spontaneous remissions, and it has been found that the degree of splenic infection is a good indication of the degree of general infection, and can be used to assess the efficiency of treatment. The hamsters were treated with 2-hydroxy stilbamidine kindly supplied by Dr. A. J. Ewins, F.R.S., because it had been observed (Fulton, 1944) that this drug in suitable doses was an effective therapeutic agent.

MATERIAL AND METHODS.

Parasitological and Chemotherapeutical.

The strain of *L. donovani* used was isolated from an Indian patient in 1939 and has since been maintained by passage in hamsters.

72 animals varying in age from 3–9 months were used. They were inoculated i.p. with a suspension of the pulped spleen of hamsters heavily infected with *L. donovani*. Each received approximately 9 million parasites and was then housed in a separate cage. A number of animals were killed without treatment at the end of 2, 4 and 6 weeks after inoculation, counts being made of the number

of parasites present in spleen smears, and histological and other data obtained for each animal. Culture of the organ was necessary to detect infection during the early stages of the disease, as parasites were not always seen in stained smears. At 6–7 weeks after inoculation a number of the remaining animals were laparotomized, and smears and cultures were made from removed splenic tissue in order to establish the degree of infection. Treatment with 2-hydroxy stilbamidine was begun five days later. All but three of the treated animals received a total of 10 doses of 20 mg. per kilo of drug given i.p. on alternate days. These three animals were killed after receiving 5 doses. The others were killed at 10, 17, 24, 31 and 46 days after the end of treatment, the last batch being killed 109 days after inoculation. With each batch a similar number of untreated controls was also killed. The necessary data on the spleen were then collected. The weight of each animal at the beginning and end of the experiment was also recorded. As some of the infections were scanty six weeks after inoculation, 12 animals were not laparotomized till 8–9 weeks after inoculation, and treatment was then carried out as before. Three of this batch died during treatment, and the others were killed at intervals of 9, 16, 31 and 44 days after it ended, the last batch 122 days after inoculation. Three normal animals were treated with drug to determine if it had any effect on the spleen.

Histological.

After being weighed and measured, the spleens were fixed in formol-mercuric chloride mixture (10 per cent of commercial formaldehyde in saturated aqueous solution of mercuric chloride—Lendrum, 1941). When fixed they were split longitudinally and paraffin sections cut. These were stained by haemalum and eosin as a routine, and further sections were stained as required by iron haema-

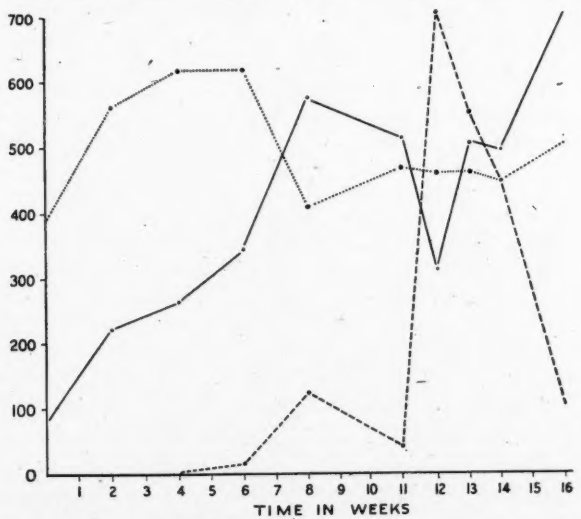

FIG. 1.—Untreated: Weight of spleen in mgs. ———
Diameter of Malpighian bodies in μ ------
Parasites per field – – – – –

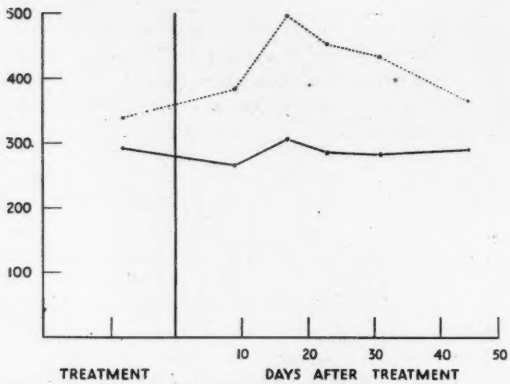

FIG. 2.—Treated : Weight of spleen in mgs. ————
Diameter of Malpighian bodies in μ - - - - - - -

toxylin and van Gieson, Masson's trichrome stain, Foote's reticulum stain, and Perl's method for free iron.

In each spleen the Malpighian bodies were measured. The two largest of those which were fairly cut across and were most nearly circular were selected. They were measured in two diameters by means of a calibrated ocular and the result expressed as the mean of the four measurements. This figure does not represent the mean diameter, but it gives a figure which can be used for comparison. Eleven healthy hamsters were killed as controls in order to establish normal measurements and for histological comparison. These animals are included in Table I and Fig. 1.

TABLE I.

Untreated.

No. of animals.	Survival after infection (days).	Mean splenic weight (mg.).	Mean splenic length (mm.).	Malpighian bodies, mean diameter (μ).	Pulp congestion.	Pulp hyperplasia.	Siderosis.	Infection.*
11	0	81	27	392	−	−	−	−
4	14	220	33	560	−	±	−	1+ 3−
4	28	259	34	617	±	±	−	+ (1·6)
3	42	338	37	616	±	+	−	+ (13)
3	56	576	42	410	−	++	−	+ (120)
3	70–77	513	40	468	−	++	+	+ (38)
3	77–84	312	33	458	−	++	+	+ (717)
3	91	511	40	462	−	++	+	+ (550)
2	98	495	36	446	−	++	+	+ (450)
3	112	707	39	506	−	++	+	+ (100)

Treated.

No. of animals.	Survival after start of treatment (days).	Mean splenic weight (mg.).	Mean splenic length (mm.).	Malpighian bodies, mean diameter (μ).	Pulp congestion.	Pulp hyperplasia.	Siderosis.	Infection.*
5	10	291	32	337	−	+	+	−
6	30	263	32	382	±	±	+	−
5	37	304	29	493	+	±	+	−
2	44	235	28	451	+	−	+	−
3	51	279	27	433	−	−	+	−
2	66	289	28	363	−	−	+	−

* Figures in brackets = number of parasites per field.

RESULTS.

The results are summarized in Table I and Figs. 1 and 2. In untreated hamsters after infection, the spleen size appears to increase up to about eight weeks, and then remains steady. It is probable that the variation which we observed after this was largely fortuitous, due to the small number of animals in each group. The earliest histological change seen was an overgrowth of the reticulum cells of the germinal centres of the Malpighian bodies causing the latter to enlarge. The overgrowth was visible two weeks after infection, and appeared to reach a maximum in four to six weeks ; after this time it became less marked. In a few cases this reticulum cell hyperplasia occurred in foci instead of being confined to the germinal centres. In the pulp the earliest change was a slight, diffuse swelling and proliferation of the reticulum cells visible after two weeks' infection. This was followed by a transitory phase of congestion lasting from the fourth to the eighth week. Between the sixth and twelfth weeks there was a progressive proliferation of the reticulum cells. This commenced in small foci which enlarged and became virtually confluent, crowding out the blood and leaving the pulp almost wholly cellular. Haemosiderin was demonstrable as brown pigment from the eleventh week onwards. Leishmania were demonstrated in direct smears in every case from the fourth week onwards, and in general the numbers increased with the age of the infection up to 12 weeks, after which they declined, but still remained above 100 per field. Leishmania were also visible in sections of spleen, and occurred in foci of hyperplasia both in the Malpighian bodies and the pulp, but were more frequent in the latter site.

In the treated series the infection was successfully eradicated, as judged by splenic smears and cultures, in all but six of the animals for periods up to 66 days after the start of treatment. The treatment was begun after 46 days of infection in one half of the animals and after 60 days in the other half. At this period the average weight of the spleen as estimated from the untreated series would be between 350 mg. and 575 mg. Yet after only half of the course of treatment the mean splenic weight was 291 mg. This implies that treatment causes a rapid reduction in size. It is, however, interesting to note that complete treatment and even 46 days of survival afterwards did not significantly alter the weight of the spleen, and that it remained around 300 mg. as compared with a normal mean of 81 mg. This persistent splenomegaly was not accounted for by cellular hyperplasia or by congestion, nor was there any increase of fibrous tissue or argentophil reticular fibres. From a histological study it appears that during the phase of active infection the whole spleen has to grow in order to contain the mass of reacting reticulum cells, and that when this reaction disappears the spleen fails to shrink to its original size.

In the six animals in which treatment failed to eradicate the infection, it did nevertheless control it because the degree of splenic reaction was considerably less than in the corresponding controls.

DISCUSSION.

The present histological findings in the spleen of untreated hamsters infected with *L. donovani* agree with the descriptions of other authors who have shown that they are similar to those occurring in man. This fact suggests that the changes which occur in the spleen as the result of treatment may also be comparable in the two cases. In man, the enlarged spleen of the kala-azar patient,

as in the case of the hamster, undergoes reduction in size as the result of effective treatment. In his case the prognosis in untreated cases is serious, and the mortality-rate is very high with only a small percentage of spontaneous recoveries. In the hamster there is no spontaneous regression of the disease. Our results with untreated hamsters suggest that there is a rapid and continuous increase in splenic size as the result of the infection during the period of observation. The decrease noted at the twelfth week after inoculation we regard as fortuitous, due to the small number of animals used, and the decrease indicated in the intensity of infection in the later stages is ascribed to the same cause, as it was not observed in previous experiments where, however, the other observations pertinent to the present study were not made. It is well recognized, moreover, that the degree of infection in animals receiving the same inoculum of infective material may, for some reason unknown, show wide variations at any particular time. In the animals effectively treated, the spleen weight remained fairly constant above that of the uninfected controls, but was much less than that of the infected animals which received no treatment. The size of the Malpighian bodies showed similar variations. The spleen of the animals, presumably cured, did not revert to normal size, but as a result of the cellular reactions which had occurred, was still enlarged at the end of the observation period. In view of the fact that the hamsters received a large inoculum of infective material and that parasites were shortly afterwards demonstrable in the viscera, it may be unwise to conclude that the sequence of events in the hamster spleen follows closely that which occurs in the case of man.

SUMMARY.

A batch of 72 golden hamsters was used to study the histological changes in the spleen following infection with *L. donovani* and the changes which occur in this organ as the result of treatment by a therapeutic agent.

It was found that in untreated animals the size of the spleen and its Malpighian bodies was considerably greater than in normal uninfected controls. The size of the organ was markedly reduced by treatment, but remained consistently greater than that of normal uninfected controls during a period of approximately nine weeks' observation after the end of treatment.

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IMMUNITY TO HOMOLOGOUS GRAFTED SKIN. I. THE SUPPRESSION OF CELL DIVISION IN GRAFTS TRANSPLANTED TO IMMUNIZED ANIMALS.

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IT is a well-established empirical fact that skin does not survive transplantation between individual rabbits or individual human beings of ordinary genetic diversity. Skin so grafted will at first behave "normally," i.e., like a graft taken from some other part of the same individual; but in due course it is invariably destroyed. The transplantation of skin between about 900 distinct pairs of laboratory rabbits has failed to provide any exception to this rule.

The evidence summarized in the paragraphs which follow is taken from papers by Gibson and Medawar (1943), Medawar (1944), and Medawar (1945).

Skin homografts transplanted to a rabbit which has earlier received and reacted against grafts from the same donor source behave in a manner quite distinct from their predecessors; for (*a*) they are destroyed more rapidly, (*b*) the manner by which they are vascularized and invaded by native leucocytes is profoundly modified, and (*c*) the hyperplasia of the epidermal epithelium that is the invariable rule with autografts and normal ("1st-set") homografts is in these "2nd-set" homografts completely suppressed. The object of this paper is to provide numerical evidence for the third of these statements, and to examine the specificity of the reaction by means of which epidermal proliferation is brought to a standstill.

The following experimental plan was adopted. First, skin was grafted in high dosage from a rabbit D-1 to a rabbit R. Six days later a graft sample was removed and sectioned to provide an estimate of the frequency of cell-divisions within the epidermal epithelium. Six to nine days after this biopsy the grafts of first planting were found to have been destroyed. One further graft from the original donor D-1 and one graft from a new donor D-2 were then transplanted to a fresh operation field on the recipient R. Six days later the two grafts of second planting were in their turn removed and examined for mitotic figures.

Six independent experiments of the form described above were done. Each provided a strictly-controlled comparison between the frequency of mitoses in 1st- and 2nd-set grafts, and made it possible to determine the specificity of the reaction of mitotic inhibition towards the skin of the original donor D-1. A 6-day period from grafting was chosen as the sampling interval, because earlier work had shown that the outburst of cell division in the epidermis of autografts and 1st-set homografts would be well established within it. It will be noted that the 1st-set grafts were transplanted in higher dosage than those of the second set. This disparity will tell *against* the 1st-set grafts, because, other things being equal, the survival time of a homograft varies inversely with the systemic graft dosage. The disparity of dosage therefore makes the recorded superiority of 1st-set grafts proportionately more convincing.

METHODS.

The rabbits used in these experiments were adults of both sexes, 2–2½ kg. in weight, taken from heterogeneous dealers' stocks. The general technique of grafting has been described in full by Medawar (1944), and the special variant used in these experiments by Medawar (1945). The term "large pinch graft" is used in a technical sense: estimates by Medawar (1944) gave the "large" pinch graft a mean diameter of 8·5 ± 0·3 mm. after cutting and fixation, but before planting, and a modal average weight class of 0·045–0·055 g. (Here and below, the error function attached to numerical estimates is the standard sampling error of the mean.)

Transplantation of the 1st-set (immunizing) homografts.

After clipping the outer aspect of the thigh with fine scissors, washing the exposed skin surface with soft soap and rinsing with surgical spirit, eight large pinch grafts were cut from the donor D-1 and transplanted to the skin overlying the right thoracic wall of animal R. Each graft was carefully fitted into a recipient area just large enough to receive it ("fitted grafts": Medawar, 1945). The operation field was dusted with sterile sulphadiazine powder, covered with a square of vaseline-soaked fine-mesh gauze, and wound first with plain and then with plaster-impregnated bandage. Six days later, any one of these eight grafts of first planting was removed, without the tissue surrounding and underlying it, by careful dissection from the graft bed.

Transplantation of the 2nd-set grafts.

The grafts transplanted at the first grafting operation did not survive beyond the 15th day; those borne by the majority of the animals did not survive so long. Between the 12th and 15th days, therefore, one large graft from the original donor D-1 and one large graft from a new donor D-2 were fitted to the skin overlying the *left* thoracic wall of the recipient R. Dressings were applied in the same manner as before. Six days after this second operation the two 2nd-set grafts were in their turn removed. They differed markedly to outward appearance. The 2nd-set graft derived from the original donor D-1 was in all cases hard and discoloured to a shade of yellowish-brown; generally its circumference was found to have disengaged from the surrounding native epithelium. The 2nd-set graft from the new donor D-2 did not differ markedly from a 1st-set graft: its rim was secure, and whatever swelling it showed was of the soft, freshly oedematous type. These differences are duly reflected in the respective estimates of the frequency of epidermal cell-division within them. They may be contrasted with the *complete* uniformity of all the grafts transplanted on the same occasion from a single donor: such grafts are outwardly and inwardly indistinguishable, whatever their positions on the body or their local dosages in those positions (Medawar, 1945).

Histological method.

Biopsy specimens were at once fixed in Bouin's fluid for 48 hours, dehydrated in an ethyl-alcohol series, cleared in cedarwood oil followed by ligroin, and embedded in paraffin wax. A strip of not less than 10 vertical sections was thereupon cut from the median level of each graft. (The graft being oval or

circular, the median level corresponds to that of maximal diameter.) Sections of odd number from this median strip of 10 were mounted in order upon one slide, and the alternate sections, of even number, upon a second. One slide was stained with Ehrlich's haematoxylin and counterstained with aqueous orange-G and alcoholic eosin. The other was stained with Hance's (1933) variant of Heidenhain's haematoxylin, differentiated with saturated aqueous picric acid, and mounted without counterstain after thorough washing.

All the mitotic figures in the epidermal epithelium of the five alternate sections stained with Heidenhain's haematoxylin were then counted. The following two independent estimates from two 6-day 1st-set grafts were chosen at random from the writer's records :

	Section No.					
Mitoses :	1.	3.	5.	7.	9.	
Expt. 3	65	60	70	77	64	*Mean :* $67 \cdot 2 \pm 2 \cdot 9$
Expt. 7	44	53	48	44	64	*Mean :* $50 \cdot 6 \pm 3 \cdot 7$

They show that the standard error of section sampling represents a very small fraction of the mean computed from the readings for the five sections ; and in the experiments treated as a whole (Table I) the standard errors represent no more than the fraction $0 \cdot 054 \pm 0 \cdot 005$ of the values of the means to which they relate. There can be no doubt, therefore, about the adequacy of the technique of sampling.

The validity of the technique of identifying and recording mitoses is more problematical. In these analyses an epidermal cell was held to be " in mitosis " if, and only if, it showed clear-cut chromosome figures within it. No attempt was made to distinguish between " normal " and " abnormal " mitotic figures : with material ill-adapted to chromosome study (not least because the long axes of the dividing cells may lie at any angle with regard to the plane of section) any such discrimination probably introduces as many errors as it can hope to remove. (There is little difficulty in identifying the *grossly* abnormal mitosis, particularly with a metaphase figure in polar view. It is in the finer degrees of distinction that the errors of personal judgment become unduly large.)

If uninterrupted serial sections of 8μ thickness are used for counting, a telophase figure may easily be counted twice in adjacent sections. It was for this reason that alternate sections were used.

Since the grafts used in these computations varied somewhat in size (column 5 of Table I), some adjustment had to be made in the figure representing the mean number of mitoses in the median sections. Whatever the thickness of an epidermis, the majority of the cells which divide are those which are adjacent to and define the dermo-epidermal interface. The best specific estimate of mitotic frequency must therefore be that which is arrived at by dividing the mean number of mitoses by the mean length of the dermo-epidermal junction as it appears in vertical section. But in view of the great disparities between the mitotic frequencies of 1st-set and 2nd-set grafts, it was here thought sufficient to use a less accurate correction, one based upon the diameter of the graft alone. The entry in Table I under the column-heading " Mean mitoses per mm." therefore represents the mean number of mitoses to be expected in 1 mm. length of the epidermal surface, together with those of the follicular epithelium which lies in the dermal background of this 1 mm. stretch.

RESULTS.

The numerical data for six sets of strictly controlled experiments are entered in Table I. The data for the 1st-set grafts were enlarged by four additional readings (Expt. 7–10), making ten in all.

TABLE I.—*The Frequency of Mitoses in the Epidermal Epithelium of 1st-set and 2nd-set Rabbit Skin Homografts Six Days After Grafting.*

Expt. No.	Type.	Donor and recipient.	Mean mitoses per median section ± S.E.	Graft diameter (mm.).	Mean mitoses per mm.
1	D-1 → R (1st-set)	406 → 407	25·8 ± 1·4	6·8	3·8
	D-1 → R (2nd-set)	406 → 407	0	6·2	0
	D-2 → R (2nd-set)	401 → 407	0	8·8	0
2	D-1 → R (1st-set)	416 → 417	39·4 ± 3·0	6·9	5·7
	D-1 → R (2nd-set)	416 → 417	0	6·7	0
	D-2 → R (2nd-set)	413 → 417	54·0 ± 3·5	6·5	8·3
3	D-1 → R (1st set)	426 → 427	67·2 ± 2·9	6·8	9·9
	D-1 → R (2nd-set)	426 → 427	0	7·0	0
	D-2 → R (2nd-set)	403 → 427	33·2 ± 1·5	7·2	4·6
4	D-1 → R (1st-set)	431 → 432	58·4 ± 3·8	10·0	5·8
	D-1 → R (2nd-set)	431 → 432	0	7·1	0
	D-2 → R (2nd-set)	403 → 432	27·6 ± 2·1	7·8	3·5
5	D-1 → R (1st-set)	436 → 437	45·2 ± 2·6	9·3	4·9
	D-1 → R (2nd-set)	436 → 437	0	6·7	0
	D-2 → R (2nd-set)	433 → 437	24·6 ± 1·6	7·1	3·5
6	D-1 → R (1st-set)	451 → 453	83·3 ± 2·8	7·8	10·7
	D-1 → R (2nd-set)	451 → 453	0	6·0	0
	D-2 → R (2nd-set)	454 → 453	5·0 ± 0·4	6·5	1·3
7	D-1 → R (1st-set)	411 → 412	50·6 ± 3·7	7·2	7·0
8	D-1 → R (1st-set)	421 → 422	42·6 ± 1·3	6·8	6·3
9	D-1 → R (1st-set)	457 → 458	89·4 ± 2·8	6·8	13·2
10	D-1 → R (1st-set)	459 → 460	35·8 ± 0·6	7·4	4·8

The entries in column 3 are the laboratory numbers of donor and recipient animals.

Inspection of the table shows that the fact of mitotic inhibition in skin grafts transplanted to specifically immunized animals (D-1 → R, 2nd-set grafts) is demonstrated as clearly as the design of the experiment allows. In 1st-set grafts as a whole, the mean number of mitoses per mm. of epidermal surface is 7·21 ± 0·96 ; in the six used for the controlled experiments, 6·80 ± 1·15. The 2nd-set grafts transplanted from the same donors showed, by contrast, no evidence of cell division at all.

The degree of specificity of the reaction is shown by the behaviour of the 2nd-set grafts transplanted in parallel from *new* donors (D-2 → R, 2nd-set grafts.) In these there were found to be a mean number of 3·53 ± 1·17 mitoses per mm. The data do not convince one that there has been any mitotic inhibition in these grafts at all (save in Expt. 1) ; for by applying the tests appropriate to paired data, it has been found that the mean of the differences between the readings for 1st-set grafts and 2nd-set grafts from new donors (D-2 → R) itself differs from zero by a gap that might be bridged as often as once in ten random trials of this form.

A further group of experiments was now done to determine whether grafts transplanted to immunized animals show epidermal cell divisions at *any* stage in

their history. The question resolved itself into inquiring into the occurrence of mitotic figures at the 4th day from transplantation; for no skin graft of any type shows consistent epidermal proliferation before the 4th day, and the majority of 2nd-set grafts do not survive beyond the 6th (Medawar, 1944).

Rabbits were immunized by skin grafts in high dosage as before, and then regrafted between the 12th and 16th days thereafter with one further large graft from the same donor. The graft of second planting was removed on the 4th day and examined for mitotic figures in the manner described above. The results of 10 such experiments are entered in Table II. The entries in the column headed "Mitoses" represent the *total* number of mitoses discovered in the five alternate median sections upon which the estimate is based.

TABLE II.—*The Frequency of Mitoses in the Epidermal Epithelium of 2nd-set Rabbit Skin Homografts (Type D-1 → R) Four Days After Grafting.*

Expt. No.	Donor and recipient.	Total mitoses in five median sections.	Graft diameter (mm.).
11	445 → 447	1	6·9
12	448 → 450	0	8·2
13	457 → 458	0	8·9
14	459 → 460	2	7·3
15	461 → 462	1	7·9
16	469 → 470	0	8·5
17	471 → 472	0	6·7
18	473 → 474	0	9·6
19	475 → 476	1	9·8
20	478 → 479	0	9·4

It may be concluded from the evidence presented in this table that the suppression of mitosis in 2nd-set grafts is virtually complete.

DISCUSSION.

The suppression of cell-division.

The empirical fact that cell division is brought to a standstill in skin grafts transplanted to specifically immunized animals cannot be denied. It is not in itself of much theoretical importance, because the inhibition of mitosis might very well be a mere by-product of some other, primary, immune reaction. It owes its interest to the fact that 2nd-set grafts are *not* killed outright, but *do* survive in a "vegetative" condition (*i.e.*, without cellular reproduction) for a period which is almost always greater than 4 days, though usually less than 8 (Medawar, 1944). The evidence upon which this statement is founded is not merely histological: for in a set of 24 independent tests Medawar (1944) examined the viability of 1st-set and 2nd-set homografts by the simple experiment of transplanting them back from their primary recipients to their original donors. A vigorous outgrowth of epidermal epithelium from these "autohomografts"—homografts restored to their native environments to become autografts again—constituted a proof of survival hardly subject to error. Each one of six 4-day 2nd-set homografts so tested was found to contain living epithelium, and two of six 8-day 2nd-set homografts.

There is therefore a reasonable presumption that the immune state called forth by grafting foreign homologous skin is directed against the reproductive mechanism of the transplanted cells, which is evidently put out of order, rather than against the cells' "vegetative" activities, which are not immediately affected.

Collateral evidence for this theory is contained in the fuller statement of it by Medawar (1945).

The hypothesis of mitotic inhibition fails altogether to take into account certain histological peculiarities of the skin homograft reaction in rabbits. It does not explain the violent inflammatory reaction and vascular breakdown that accompanies the destruction of 1st-set homografts in rabbits (Medawar, 1944), nor the leucocytic invasion of the grafts that anticipates it. These point rather to a reaction of " sensitization." But since the quality and intensity of the inflammatory reaction vary widely from one species of animal to another, it may as a first approximation be regarded as of secondary significance. It is of peculiar violence in the rabbit—an animal notoriously susceptible to " anaphylactic " inflammation.

The idea that an immune reflex may express itself by mitotic inhibition is by no means *necessarily* inconsistent with the classical doctrine that any such reflex may ultimately be resolved into a reaction between " antigen " and " antibody." Nevertheless, all attempts to demonstrate such a reaction *in vitro* have so far failed. The genetical implications of the theory will be discussed on another occasion. For the present it may be noted that the division of a cell involves acts of synthesis other than that of co-ordinated gene replication (mitosis), and there is no evidence that the inhibition of synthesis here under discussion is specifically directed against the nuclear genes.

The specificity of the reaction.

The experimental results upon this score show that mitotic inhibition in grafts transplanted to immunized animals shows a very high degree of specificity towards the skin of the rabbit that provided the immunizing dose of grafts (Medawar, 1944, 1945). They do not show that the specificity is absolute, and it would be most surprising if it were so. For as a general rule one would expect animals D-1 and D-2 (to use the notation of the text of the paper) to share in common at least a few antigens that are not present in the recipient R. Skin transplanted from D-1 to R will therefore establish at least some degree of immunity towards skin later grafted from D-2. The experimental results are fully consistent with this interpretation. The immune reaction is highly but not completely specific.

SUMMARY.

1. Skin homografts transplanted to a rabbit which has had no previous experience of the grafting of foreign homologous skin invariably display a major outburst of epidermal cell proliferation.

2. Skin homografts transplanted to a rabbit which has received and reacted against skin earlier grafted from the same donor undergo a complete suppression of epidermal cell division. It may be proved that such grafts survive in a " vegetative " condition alone.

3. The inhibitory reaction shows a high degree of specificity towards the skin of the rabbit that provided the earlier, immunizing, dose of homografts.

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IMMUNITY TO HOMOLOGOUS GRAFTED SKIN. II. THE RELATIONSHIP BETWEEN THE ANTIGENS OF BLOOD AND SKIN.

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THE idea that there exists an intimate relationship between the forms of incompatibility revealed by blood transfusion and tissue transplantation has been more widely accepted than any concrete evidence for it allows. (Arguments for and against the theory have been summarized by Medawar, 1943.) Immunity to " transplanted " blood appears to be of two sorts : innate (as with the A-B-O antigen system of the blood of human beings and anthropoid apes), or newly acquired in response to an antigenic stimulus. The latter represents the more usual state of affairs, and it is to this category that skin transplantation immunity belongs (Gibson and Medawar, 1943 ; Medawar, 1944, 1945*a*).

The problem dealt with in this paper can be expressed in the following restricted but nevertheless workable form : do the cellular constituents of blood share antigens in common with the antigens of skin epithelium ? The case of red cells may be considered first. If red cells and skin epithelium share antigens in common, then (*a*) the grafting of foreign homologous skin should in a proportion of cases elicit *pari passu* the formation of red-cell isoagglutinins or isolysins ; and (*b*) the transfusion or grafting of red cells should confer an appreciable degree of immunity towards skin later grafted from their donor. In theory, these corollaries are equally applicable to the case of leucocytes ; but the former, (*a*), is not workable because immunity to leucocytes cannot be demonstrated by an *in vitro* reaction. Immunity to homologous grafted skin may be recognized, however, by the suppression of cell-division in skin transplanted to a specifically immunized animal and by its greatly accelerated breakdown (Gibson and Medawar, 1943 ; Medawar, 1944, 1945*a*, 1946).

Full-grown rabbits from heterogeneous stock have been used in all the experiments described in this paper.

IMMUNITY TO RED CELLS AFTER GRAFTING FOREIGN HOMOLOGOUS SKIN.

In each of 15 trials with 15 independent pairs of rabbits, skin was grafted in high dosage from a donor D to a recipient R in the manner described by Medawar (1944). After these homografts had broken down—but in no case before the 12th day from grafting—blood was withdrawn from the marginal ear vein or median ear artery of animal R by means of a pipette with a stab point, allowed to clot spontaneously, and spun for serum. At the same time about 0·2 ml. whole blood was withdrawn from the marginal ear vein of D, and at once mixed

with not less than 10 ml. 0·4 per cent sodium citrate in 0·85 per cent NaCl solution. The blood cells were spun down, taken up in citrate saline, again spun down, and finally resuspended in about 10 ml. normal saline.

Agglutination test.

One drop of serum from R was mixed in a standard agglutination tube with two drops of the " 1 per cent " saline suspension of blood cells from D. (Slide tests, at first done in parallel with the tube tests, were early abandoned.) The tubes were incubated for 4 hours at 38° C. before their first examination, and left overnight at room temperature before their second. Microscopical examination was made in all trials, though due attention was paid to the nature of the packing and cohesiveness of the red-cell sediment.

No trace of agglutination or haemolysis was recorded in any of these tests. For the sake of completeness it should be added that two of the recipient animals were subjected to "adjuvant" immunization with native chicken plasma. The chicken plasma precipitin titre on the day of the agglutination test was greater than 1000.

A second test made use of a group of 5 rabbits, each one of which received a graft from each one of the others, and from 19 other rabbits as well. (These were animals Nos. 1–5 of the cross-grafting test described in full by Medawar, 1945*a*, pp. 165–168). Fifteen days after this multiple grafting operation the serum of each animal was tested in the manner described above against the red cells of the other four. This experiment, like its predecessor, gave uniformly negative results. It is clear that the formation of red-cell agglutinins is not a normal accompaniment to the reaction provoked by the grafting of foreign homologous skin.

IMMUNITY TO SKIN HOMOGRAFTS FOLLOWING TRANSPLANTATIONS OF BLOOD CELLS.

The four experiments now to be dealt with represent the converse of those described in the preceding section, their object being to determine whether the transplantation of blood cells from one rabbit to another confers any degree of immunity towards skin later grafted from the same donor. The experiments are reported in the order in which they were done, and they take the following variables into account: the route of the blood cell injection, the type of blood cell, and the cellular dosage.

To test for the existence of a " skin immune state " the blood-cell recipient was grafted, after an appropriate interval of time, with one large pinch graft (modal average weight 0·045–0·055 g., mean diameter of freshly cut grafts after fixation, 8·5 $\pm$ 0·3 mm.) from the blood cell donor. The graft was cut from the outer aspect of the thigh and accurately fitted to the skin overlying the right thoracic wall (Medawar, 1945*a*). Alternative courses were then followed. In Expts. 1 and 2 the graft was allowed to remain in residence for 12 days, and then removed and sectioned to provide a diagnosis of its degree of survival. In Expts. 3 and 4 the graft was removed on the 6th day for mitotic counting, using the technique described in full in the first paper of this series. The second test is the more delicate: it became possible to use it only when the experiments described in the first paper of this series were complete.

First Experiment.

Each of the five similar tests that constitute this experiment made use of three rabbits: blood and skin donor (D), red-cell recipient (R-1), and white-cell recipient (R-2).

Not less than 5 ml. whole blood from the median ear artery of rabbit D was received into a test-tube through a hard glass cannula with a sheared-off stab point (fig. 1*a*). A measured 4 ml. was then at once withdrawn and mixed with 0·4 ml. 4 per cent Na citrate in the fractionation tube illustrated by fig. 1*b*. The fractionation tube was spun, with the central withdrawal tube in position and sealed with wax, until the leucocytes formed a compact layer (the "buffy coat") between the red cells and supernatant plasma. After withdrawing the plasma carefully through the side hole of the outer tube the wax seal on the inner tube was broken, and one-fourth of the cellular deposit, consisting of pure red cells, was withdrawn by means of a long fine pipette and mixed with one-half of the decanted citrate plasma. The pure red-cell suspension, containing the red cells of 1 ml. whole blood, was thereupon injected into the marginal ear vein of animal R-1.

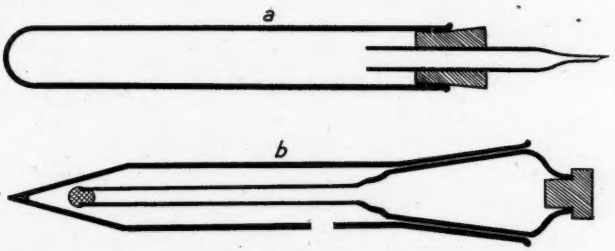


FIG. 1 (*a*).—Test-tube and cannula with sheared-off stab point for collecting blood directly from the median ear artery of the rabbit. A narrow slot is cut in the cork to allow the escape of displaced air. 1 (*b*).—Fractionation tube made from standard ground glass apparatus (B-19 joint). Calibration marks are not shown. See text.

Second and third fractions of the cellular sediment, together comprising one-half of its total volume, were then withdrawn as before and discarded. The final fraction, containing the red cells of 1 ml. whole blood together with the leucocytes of 4 ml. whole blood, was mixed with the remaining half of the decanted citrate plasma and injected into the marginal ear vein of animal R-2.

The two injections were repeated on the 3rd, 6th, and 9th days, so that each animal received a course of four injections in all. On the 16th day the sera of R-1 and R-2 were tested for donor red-cell agglutinins. None were expected and none were found. Two "large" pinch grafts were then cut from D, one being transplanted to R-1 and the other to R-2. Twelve days after grafting—28 days after the first injection—the grafts were removed, together with the tissue surrounding and underlying them, for histological examination. (It was clear to outward inspection that many had so far survived the homologous transplantation.) The histological reports are based upon the study of adjacent 8μ sections cut from not less than 5 equally-spaced vertical levels in the graft and stained with Ehrlich's haematoxylin, orange-G and eosin, and with Heidenhain's haematoxylin and picro-fuchsin respectively.

The results set out under the column-heading "Survival" in Tables I and II make use of the following notation (Medawar, 1945*a*):

1 = Complete survival of epidermal epithelium.
$\frac{3}{4}$ = The greater part of the epithelium surviving.
$\frac{1}{2}$ = About one-half of the epithelium surviving.
$\frac{1}{4}$ = The greater part of the epithelium destroyed.
0 = Total breakdown.

The propriety of basing a diagnosis of cellular survival upon histological evidence has been discussed in full by Medawar (1944). The "mark" awarded in the last column of the table is based upon a comparison between the pair of specimens afforded by each test: the mark "+" is awarded if the degree of survival in the R-1 graft is higher than in the R-2, "=" if it is the same, and "−" if it is less.

TABLE I.—*The Results of Strictly Paired Experiments Illustrating the Degree of Survival after* 12 *Days of Skin Homografts Transplanted to Rabbits Previously Injected through the Intravenous Route with Red Cells* (*R*-1) *and Red Cells* + *Leucocytes* (*R*-2).

Test No.	Donor.	Red-cell recipient (R-1).	Survival.	Leucocyte recipient (R-2).	Survival.	Mark.
1	383	384*	1	385*	1	=
2	388	389	$\frac{1}{2}$	390	1	−
3	393	394	1	395	0	+
4	403	404	0	405	$\frac{1}{2}$	−
5	408	409	1	410	0	+

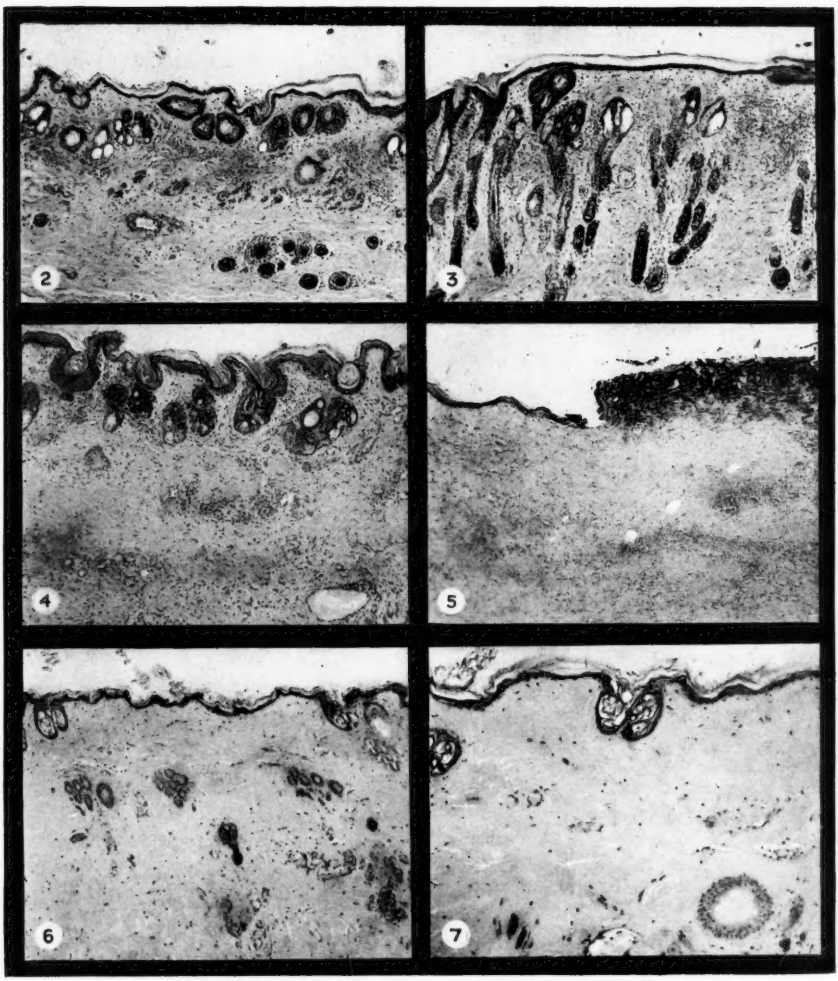
* Fig. 2 and 3.

(*All photographs are of sections stained with Ehrlich's hæmatoxylin, orange-G, and eosin.*)

FIG. 2 and 3.—Expt. 1, test 1: 12-day homografts from rabbits injected through the intravenous route with pure red cells (Fig. 2) and red cells + leucocytes (Fig. 3). Neither graft gives any evidence of an earlier immunization of its recipient. New hairs and glandular acini have matured in both, and the epithelium shows the retrograde thinning-out that follows the burst of hyperplasia in autografts and in all homografts that survive so long (Medawar, 1944). The specific vascular and mesenchyme cell reaction that is clearly developing is likewise in every respect characteristic of homografts that have been transplanted to *non*-immunized rabbits. (× 40.)

FIG. 4 and 5.—Expt. 2, test 3. Fig. 4 illustrates a 12-day homograft from a rabbit injected intradermally with a saline suspension of pure red cells. The condition is similar to that illustrated by Figs. 2 and 3, save that the hyperplastic condition of the superficial epithelium is more strongly marked and new hairs have not yet matured. By contrast, Fig. 5 illustrates a 12-day homograft from a rabbit which had received intradermal injections of pure leucocytes. Breakdown is of such long standing that native epithelium (seen on the left of the photograph) has begun to invade and overgrow the deeper levels of the graft dermis. (Fig. 4 × 40; Fig. 5 × 30.)

FIG. 6 and 7.—Expt. 4, test 4, illustrating in low and higher power views respectively the condition of a 6-day homograft from a rabbit immunized by a single intradermal injection of the leucocytes of 4 ml. whole blood. The epithelium of the graft is in a surviving condition and gives some evidence of general amoeboid activity (e.g. the upward migration of follicle epithelium towards the surface). Nevertheless cell division has been almost wholly suppressed: the epidermis is thin and unstratified and the cuticle remains delicate. (Fig. 6 × 40; Fig. 7 × 84.)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7

Reference to the table shows that the number of grafts showing *any* degree of survival was 4 out of 5 in the case of red-cell recipients and 3 out of 5 in the case of leucocyte recipients. On this score there is nothing to choose between them. The question of whether recipients of either class were to any degree immunized by the blood cell injections may be answered with reference to two independent groups of controls. Of 20 normal untreated rabbits which carried skin homografts in exactly similar dosage to that used in the experiments just described, 13 still bore surviving foreign epithelium on the 12th day (Medawar, 1945*a*). On the other hand (Medawar, 1945*a*), there is only one instance in 30 independent trials of a skin homograft surviving 12 days after transplantation to an animal immunized by an earlier grafting of skin from the same donor. The experimental results recorded here obviously belong to the former of these two categories ; there is no evidence of any immunization whatsoever.

This conclusion is decisively confirmed by the histological appearance of the experimental grafts. All of them showed either a typical generalized epidermal hyperplasia (Fig. 2 and 3) *or* evidence (e.g. visible acidophilic remains of hyperplastic follicle epithelium, multilayered cuticle, etc.) that hyperplasia had set in before the process of breakdown. Grafts transplanted to specifically immunized animals *never* undergo hyperplastic changes (cf. the first paper of this series). Moreover, three of the R-1 grafts and two of the R-2 grafts had progressed so far as to form new hairs (Fig. 2 and 3)—a state of affairs never once observed in grafts transplanted to immunized animals. Finally, the pattern of vascularization and the nature of the invasion of the grafts by leucocytes of native origin were in all respects characteristic of homografts transplanted to non-immunized animals.

Second Experiment.

In the second experiment the blood cells were introduced through the *intradermal* route. Although the experimental plan was in the main similar to that just described, the red-cell and leucocyte recipients were paired through a common donor in only three of the trials. The following additional modifications were made : (*a*) the cells were suspended in 2·0 ml. normal saline, and not in citrated plasma, before injection ; (*b*) only 0·1 ml. of the sedimented pure red-cell concentrate was administered to the R-1 animals, to reduce the disparity between the numbers of red cells and leucocytes injected ; (*c*) the leucocytes crudely separated by centrifugation in the manner already described were almost wholly freed from red cells by a second and, where necessary, a third centrifugation in tubes of 2 mm. diameter.

The injections were made at 2-day intervals, the R-2 rabbits receiving a course of three injections (and thus the leucocytes of 12 ml. whole blood in all) and the red-cell recipients a course of four. The cell suspension was on each occasion distributed over an average of 10 intradermal " blebs " spaced not less than 2 cm. apart on the shaved skin overlying the right thoracic wall. Care was taken to ensure that the suspension entered the compact collagenous tissue of the dermis, and not the looser " splitting " layer of connective tissue between the true dermis and the panniculus carnosus. The leucocyte injection-points in some animals came to be marked by small hard palpable nodules ; and the injection of red cells was found in some animals, but not in all, to provoke a rapid growth of coarse hair.

Not less than one week after the last injection, one large pinch graft was transplanted from the blood donor to the recipient(s). The grafts were removed for examination on the 12th day thereafter. The results of the experiment as a whole are set out in Table II, which makes use of the same notation as Table I.

TABLE II.—*The Results of Experiments Illustrating the Degree of Survival After 12 Days of Skin Homografts Transplanted to Rabbits Previously Injected through the Intradermal Route with Red Cells (R-1) and Leucocytes (R-2).*

Test No.	Donor.	Red-cell recipient (R-1).	Survival.	Leucocyte recipient (R-2).	Survival.
1	413	414	$\frac{1}{4}$	415	0
2	418	...	...	420	0
3	423	424*	1	425*	0
4	428	429	$\frac{3}{4}$	430	0
5	433	...	...	435	0
6	438	439	$\frac{1}{4}$	...	...
7	438	440	0	...	...

* Fig. 4 and 5.

As before, two questions may be asked: Do the R-1 and R-2 animals differ in their responses? And if so, does either group give any evidence of immunization by blood cells? It is evident that the R-1 group has a significantly higher proportion of grafts (4 in 5) bearing some fraction of surviving foreign epithelium than the R-2 group (0 in 5), for mere luck of sampling would not be expected to reveal so severe a bias against the R-2 grafts more often than once in 42 random trials. It is equally clear that the R-1 group, with four surviving grafts in five, is virtually indistinguishable from the control group of 20 animals already referred to, in which 13 grafts were found to have survived. There is no evidence, therefore, that the intradermal injection of red cells in this dosage confers any immunity towards skin later grafted from the same donor. The dosage was small, and in no case was the formation of donor red-cell agglutinins observed; but it was of the same order of magnitude as that which, in the case of skin, is already known to be fully effective.

The leucocyte recipients, by contrast, give the clearest evidence of immunization: the disparity between the proportions of animals bearing surviving grafts in the experimental set (0 in 5) and in the control set (13 in 20) is such as might be expected to occur, by luck of sampling alone, not more often than once in 67 random trials. Histological evidence reinforces this conclusion (fig. 5). Breakdown in the grafts carried by R-2 animals was of such long standing that the surrounding native epidermis had in many cases begun to undermine and nip off the superficial layers of the homograft dermis (cf. Medawar, 1945*a*); the broken-down remains of the homograft epithelium gave no evidence of having at any earlier time undergone proliferation; and the primary blood vessels of the grafts had long since broken down and been replaced by the multiple capillary invasion of the dermis that constitutes a homograft's second cycle of vascularization (Medawar, 1944, 1945*a*). The grafts carried by the red-cell recipients were in all the properties described at the end of the section devoted to Experiment 1 indistinguishable from homografts transplanted to non-immune animals (*cf.* fig. 4).

Third Experiment.

In this experiment an attempt was made to decide whether really massive intravenous injections of whole blood conferred any degree of immunity towards skin later grafted from the blood donor. The diagnosis of the "skin-immune" state was made by the technique of mitotic counting described in the first paper of this series.

The experiment comprises five independent trials, each one making use of an independent pair of animals—donor (D) and recipient (R). An average volume of just over 12 ml. whole blood was withdrawn on each of six occasions at 2-day intervals from the median ear arteries of the donor, and received directly in to a tube containing 1·0 ml. of 4% Na citrate. With the least possible delay, the citrated blood was on each occasion transfused into any one of the ear veins of rabbit R, which received not less than 72 ml. whole blood in all. Nine days after the last injection (19 after the first) the serum of R was tested for red-cell agglutinins, and a single large pinch graft from the donor was fitted to the skin overlying the right thoracic wall. This graft was removed on the *sixth* day for the standardized routine of mitotic counting, and the serum of the recipient was for a second time tested for agglutinins.

TABLE III.—*The Results of Five Similar Tests Illustrating the Degree of Epidermal Proliferation After 6 Days in Skin Homografts Transplanted to Rabbits which had Earlier Received Massive Intravenous Injections of Whole Blood.*

Test No.	Donor and recipient.	Mean mitoses per median section ± S.E.	Graft diameter (mm.).	Mean mitoses per mm.	Agglutination test. 19 days.	Agglutination test. 25 days.
1	442 → 444	33·7 ± 2·2	6·9	4·9	0	0
2	445 → 446	35·8 ± 2·0	7·3	4·9	++	+
3	448 → 449	11·8 ± 0·7	7·5	1·6	0	0
4	451 → 452	10·0 ± 1·6	9·0	1·1	0	0
5	454 → 455	56·2 ± 3·4	7·5	7·6	0	0

The results of five such tests are summarized in Table III. It is evident that mitotic activity has not been wholly suppressed, and the grafts did indeed give straightforward histological evidence of some degree of epidermal hyperplasia. The mean of the entries in the column headed "Mean mitoses per mm.," with its standard error, is 4·00 ± 1·19; whereas the mean for homografts transplanted in high dosage to non-immunized animals had earlier been found from 10 independent 6-day estimates to be 7·21 ± 0·96. (The difference of dosage will tell against the control set, if it has any effect at all; for the survival time of homografts varies inversely with the graft dosage.) The recorded difference between the means derived from these two samples is such as might be expected to occur only once in 20 random drafts from the same normal population; so it is not unreasonable to infer that there has been some degree of immunization. Tests on a larger scale would be required to put the matter beyond reasonable doubt. The conclusion that "immunity to skin homografts following massive intravenous transfusions of homologous whole blood is either trivial or absent" does, however, appear to be warranted by the data.

It is curious that red-cell agglutinins developed in only one animal (446, test 2). Their occurence evidently bore no relationship to the mitotic frequency in the corresponding graft.

Fourth Experiment.

The second experiment showed that three intradermal injections of the leucocytes of 4 ml. whole blood were sufficient to establish a complete immunity towards skin later grafted from the same donor. In this section it is shown that a high but not complete degree of immunity may be generated by one such intradermal injection alone. Apart from the fact that only one intradermal injection was administered, the plan of this experiment differed from that governing the leucocyte recipients of Experiment 2, in that (*a*) the technique of mitotic counting was used for the diagnosis of the skin-immune state, as in Expt. 3; and (*b*) a different method of leucocyte fractionation was employed. After dilution with an equal volume of 0·8 per cent Na citrate in 0·85 per cent NaCl, the 4 ml. whole blood was spun so gently as to leave the majority of the leucocytes in suspension. The supernatant fluid was decanted and the volume of the blood restored to its original value by adding isotonic citrate saline. After spinning gently for a second and a third time, the leucocyte-containing supernatant fluids were combined and briskly spun down. The pure leucocyte sediment—not always easy to disperse—was finally taken up in 2·0 ml. normal saline, and injected in the form of ten "blebs" into the dermis of the skin overlying the right thoracic wall of the recipient R. Sixteen days after this injection animal R was grafted with the standard dosage of skin from D; and six days after transplantation the graft was removed for mitotic counting.

This experiment is thus directly comparable with Expt. 3. Its results are summarized in Table IV.

TABLE IV.—*The Results of Five Similar Tests Illustrating the Degree of Epidermal Proliferation After 6 Days in Skin Homografts Transplanted to Rabbits which had Earlier Received Small Intradermal Injections of Leucocytes.*

Test No.	Donor and recipient.	Mean mitoses per median section ± S.E.	Graft diameter (mm.).	Mean mitoses per mm.
1	469 → 477	16·4 ± 2·1	9·0	1·8
2	473 → 480	41·0 ± 1·7	8·9	4·6
3	481 → 483	27·8 ± 1·9	7·5	3·7
4	484 → 486*	4·2 ± 0·5	8·7	0·5
5	478 → 487	0	8·4	0·0

* Fig. 6 and 7.

The mean of the entries in the column headed "Mean mitoses per mm." is 2·21 ± 0·87; that derived from the set of ten controls already cited in the discussion of Expt. 3, 7·21 ± 0·96. There can be no question of the significance of this difference: a single intradermal injection of the leucocytes of 4 ml. whole blood produces a high degree of immunity towards skin later grafted from the blood donor. It is not a complete immunity, for if it were so the mitotic frequency would have dropped to zero (see the first paper of this series). But it is certainly not less than that called forth by the *intravenous* transfusion of 72 ml. whole blood (Expt. 3); i.e. of a quantity of blood representing 18 times the leucocyte dosage.

Fig. 6 and 7 illustrate in low- and higher-power views respectively the condition of the epidermis in the graft removed from animal No. 486. The cells were clearly "alive" before fixation, but there is little indication of epidermal hyperplasia.

DISCUSSION.

The role of leucocytes.

The fact that leucocytes injected intradermally will immunize their recipient towards skin epithelium later transplanted from their donor shows that these two " tissues " share antigens in common. The " skin-immune " reaction is not, therefore, rigidly tissue-specific. The fact that the operative dose of leucocytes is of the same order of magnitude as that of a sufficient immunizing dose of skin suggests that the two tissues share a good many antigens in common. There is no evidence that they share *all* their antigens in common, and experiments of the type described here could not provide it.

Leucocytes are of purely mesenchymal origin. Since skin grafts carry with them a complement of dermal mesenchyme cells, it might be argued that the immunizing effect of leucocytes is exerted directly against them, and only indirectly against the homologous skin epithelium. This argument is untenable because (*a*) every test that has been used for the quantitative diagnosis of the " skin immune state " is based upon the reactions of epidermal epithelium alone ; and (*b*) these reactions are not significantly influenced by the presence of dermal mesenchyme. The pure epidermal epithelium which grows outwards from skin homografts transplanted to the middle of a widespread raw area responds in just the same manner as the " resident " epithelium on the surface of the graft itself (Gibson and Medawar, 1943 ; Medawar, 1944).

The route of injection.

The immunizing power of leucocytes towards skin is *at least* 18 times more effective through the intradermal route than through the intravenous. It will be noted that, whereas " every intradermal injection is truly intralymphatic " (McMaster and Kidd, 1937), a properly executed intravenous injection is made at just that point which is physiologically most remote from the lymph nodes (Expt. 3). The experiments reported here do not therefore conflict with the view that lymph nodes play a part in the manufacture of antibodies (McMaster and Hudack, 1935 ; Ehrich and Harris, 1942, 1945), though they can hardly be said to support it strongly.

A wide disparity between the effectiveness of the intradermal and intravenous routes of injection has already been observed in studies on the sensitization of skin to chemical compounds (Sabin and Joyner, 1938 ; Landsteiner and Chase, 1939).

The role of red cells.

In planning these experiments, it was not assumed that the antigens which red cells might share with skin were necessarily those that can be made to express themselves as iso-agglutinogens. It proved, however, that doses of red cells very much greater in bulk and cell number than those which are effective when skin itself is the immunizing agent did not confer skin-immunity of a type appreciable by the methods here described. Nor, conversely, did the grafting of massive doses of skin elicit the formation of homologous red-cell agglutinins. This second group of observations confirms the experience of Kozelka (1933), and Haddow (1934). On the other hand, Gorer (1937, 1938) discovered that a red-cell agglutinogen of Strong A-line mice was identical with one of two or

three antigens which generate immunity towards a sarcoma arising in the same line when it is transplanted to a host that lacks them. (This terminology is not that adopted by cancer research workers, who speak as a rule of genetic "factors for susceptibility.") It follows from Gorer's experiments that tissue cells and red blood cells may sometimes share antigens in common; but the practical consequences of this fact, so far as skin immunity is concerned, are evidently negligible.

Leucocytes and blood transfusion.

The behaviour of leucocytes is commonly neglected in transfusion practice, although fresh whole blood and blood of short storage certainly contains viable white cells. This neglect seems to be empirically well founded. The results of Expt. 3 in the text suggest that no significant leucocyte immunity would develop in response to transfusion through the intravenous route, though it might do so if the sternal route were used. The only clinically relevant effect that leucocyte immunity might be expected to exert is to prejudice the success of skin homografts when used as temporary wound dressings (Medawar, 1945*b*). This effect is of such a special character that it can obviously be neglected in transfusion practice.

SUMMARY

1. The transplantation of massive doses of skin from one rabbit to another does not elicit the formation of red-cell iso-agglutinins.

2. Intradermal and intravenous injections of foreign homologous red cells confer no appreciable immunity towards skin later grafted from the red-cell donor.

3. The intradermal injection of foreign homologous leucocytes confers a typical immunity towards skin later grafted from the leucocyte donor. It is inferred that leucocytes share antigens in common with the antigens of skin, and that tissue transplantation immunity is not rigidly tissue-specific.

4. The immunizing effect of leucocytes is at least 18 times more effective through the intradermal route than through the intravenous.

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A SEROLOGICAL INVESTIGATION IN HEPATITIS USING THE COMPLEMENT-FIXATION REACTION.

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In this report an account is given of serological investigations using the complement-fixation reaction on the sera of patients with infective hepatitis, homologous serum hepatitis and post-arsphenamine hepatitis, and on material obtained from cases of acute necrosis of the liver either of unknown aetiology, occurring in the course of an epidemic of infective hepatitis, or following treatment by plasma transfusion or with organic arsenicals. This investigation is part of a wider series of serological investigations made in an attempt to find some specific reaction for the diagnosis of infective hepatitis and the possible differentiation of types of hepatitis.

METHODS.

Preparation of liver antigens.

All types of specimen were cut up finely with scissors and ground in a mortar. A 15 per cent suspension in normal saline was then made and allowed to extract for 48 hours at + 2° C. The material was then spun for 10 minutes at 2000 r.p.m., and the supernatant was stored at — 20° C. When antigens were heated they were heated to 56° C. for 30 minutes, left at + 2° C. overnight and then spun for 10 minutes at 2000 r.p.m. The supernatant was then stored at + 2° C. Phenol was added to most specimens to a final dilution of 0·5 per cent at the time of heating. Where other processes of extraction were used they are described separately.

Technique of complement-fixation test.

Three units of complement were used, and a 3 per cent suspension of sheep's erythrocytes sensitized with five units of haemolysin. When the serum dilutions were varied those normally used were 1 : 5, 1 : 10, 1 : 20 and 1 : 40, and the antigen was used in the maximum strength at which it did not show any anti-complementary activity (this was from one-and-a-half to four times the minimum fixing dose). When the antigen dilutions were varied the serum was used in a dilution which provided twice the quantity needed for complete fixation with known positive antigens except when the titre of the serum was 1 : 5 when that dilution was used. All reagents were used in 0·2 c.c. quantities. In some earlier experiments fixation was made at 37° C. for one hour. It was, however, found that fixation at + 2° C. for 18 hours followed by one hour at room temperature gave titres three times as high as with the former technique. Fixation in the cold was, therefore, used in all subsequent experiments.

After the addition of the sensitized cells the results were read after one hour's incubation at 37° C. They were recorded in the usual manner as ++++, +++, ++, + and 0 according to the degree of fixation. In analysing the results only complete fixation is regarded as positive and +++ as doubtful. ++ and + reactions have been disregarded.

EXPERIMENTAL RESULTS.

Acute necrosis of liver of uncertain aetiology.

These livers all showed a histological picture similar to that of cases with known infective hepatitis contacts. Some of them gave extracts too anti-complementary for use. Seventeen were usable, but none of them gave a greater degree of fixation with any sera than that obtained with control antigens made from normal livers.

Fatal cases of infective hepatitis occurring during epidemics.

Antigens from five such livers were prepared. Three were tested both against sera from cases occurring in the same outbreak (mainly relations of the fatal cases) and sera from various other sources. The other two were only tested against miscellaneous sera from cases of hepatitis. One of these latter two showed fixation with five out of nine sera from cases convalescent from infective hepatitis; these five cases gave no fixation with control livers. Positive fixation was not given with sera from cases of arsenotherapy jaundice, which, however, gave specific positive fixation with antigens prepared from livers of cases of the same condition. Unfortunately only a very small quantity of this liver was available, so that no more extensive investigations could be made. The other liver antigens gave no fixation with any sera differing from that obtained with antigens made from normal livers.

Liver from cases of reputed homologous serum hepatitis.

Extracts from nine such livers were tested against sera from known cases of homologous serum hepatitis, reputed homologous serum hepatitis, infective hepatitis and post-arsphenamine hepatitis. No complement fixation differing from that with control antigens was observed.

Known icterogenic sera.

These were highly anti-complementary unheated, and had to be heated to 56° C. for 30 minutes before use. Two batches were tested against early and convalescent sera from cases of homologous serum hepatitis resulting from the injection of serum from the same batch.

No complement fixation was found either with the untreated serum or with the supernatant and resuspended deposit of one specimen after centrifugation of 15 c.c. at 30,000 r.p.m. for 30 minutes.

Post-arsphenamine hepatitis.

The results with liver antigens and sera from cases of post-arsphenamine hepatitis are in marked contrast with the negative results from infective hepatitis and homologous serum hepatitis.

During the period in which the disease was epidemic in military clinics, the serum in a high percentage of cases of post-arsphenamine hepatitis contained a complement-fixing antibody which reacted with liver extracts from cases dying of post-arsphenamine hepatitis, but not with any other liver extracts. Sera collected earlier in the disease showed far fewer positives than those collected more than eight weeks after the onset. During the epidemic period 28·4 per cent

of 67 sera collected before the 8th week gave fixation; while 67 per cent of 55 sera collected after that time were positive. A few sera gave fixation more than 6 months after the onset of the disease, and two of four tested over a year after the onset gave fixation.

The results of complement-fixation tests made with 34 pairs of early and late sera, including seven cases which have occurred since the epidemic incidence of the disease ceased, are shown in Table I, and the results of all sera, including those separately tabulated in I, are shown in Table II. They are divided into

TABLE I.—*Results of Complement-fixation Tests with Pairs of Early and Late Sera and Liver Antigens in Post-arsphenamine Hepatitis.*

	Number negative.	Number doubtful.	Number positive.	Total.	Percentage positive.
Early sera	28	0	6	34	17·6
Late sera	12	3	19	34	56

TABLE II.—*Complement Fixation in Post-arsphenamine Hepatitis. All Sera Divided into those Before and those After the 8th Week of the Disease.*

	Number negative.	Number doubtful.	Number positive.	Total.	Percentage positive.
Early sera	54	4	19	77	24·7
Late sera	21	4	37	62	59·5

sera taken before and after eight weeks from the onset of the disease. Where more than two sera from any case had been tested, only the results of the first and the last test have been used in compiling these tables.

The time at which complement-fixing antibodies first appeared varied between being present in the second week and only being found in specimens taken after the 8th week. The progress of the development of positive fixation in three cases is shown in detail in Table III. The fixation with control antigens seen in early sera from Σ82 is discussed later.

TABLE III.—*Development of Complement-fixing Antibodies in 3 Cases Followed Throughout the Disease.*

Period of the disease.	Titre of complete fixation.					
	Σ57.		Σ82.		Σ83.	
	Test antigen.	Control antigen.	Test antigen.	Control antigen.	Test antigen.	Control antigen.
2nd week	0	0	1 : 5	1 : 5	0	0
5th „	0	0	0	0	1 : 5	0
7th „	1 : 20	0	1 : 5	0	1 : 5	0
10th „	1 : 20	0	1 : 40	0	1 : 5	0

Seventy sera from 52 cases of infective hepatitis were tested against liver extracts from post-arsphenamine hepatitis. In 18 cases paired sera from early in the disease and after the 8th week were tested. Among the other 34 cases 16 of the sera were from convalescent cases and 18 from acute cases. No positives differing from those with control antigens were found. In six undoubted cases of homologous serum hepatitis tested at all stages of the disease no positives differing from those with control antigens were found. Other reputed cases of homologous serum hepatitis also gave negative results. All antigens both from infective hepatitis and homologous serum hepatitis cases were tested against known positive post-arsphenamine hepatitis sera. No positive results were obtained.

Various types of control sera were also tested without finding any which gave similar results to the post-arsphenamine hepatitis cases. A summary of the results from these controls is given in Table IV. In two instances the serum

TABLE IV.—*Results of Complement-fixation Tests with Control Sera and Liver Antigens from Post-arsphenamine Hepatitis and Normal Livers.*

Source of serum.	Number showing no fixation.	Number positive with post-arsphenamine antigens only.	Number positive with all liver antigens.	Total.	Percentage positive with all liver antigens.
Normals	76	0	1	77	1·3
3rd course arsenotherapy without jaundice	20	0	1	21	5
W.R. positive	12	0	1 (doubtful)	13	7·7
Obstructive jaundice	4	0	1	5	20
Miscellaneous conditions showing liver damage	17	0	3*	20	15

* Two of these were cases of malaria (five tested in all).

of cases receiving anti-syphilitic treatment tested as controls gave specific complement fixation with a post-arsphenamine antigen. In each case further questioning disclosed that the patients had suffered from post-arsphenamine hepatitis in the last year. None of the other 20 cases tested during the third course of arsenotherapy either admitted to having suffered from jaundice during the treatment or had any record of jaundice, and none showed positive fixation. As may be seen from the table neither normals, syphilitics with a positive Wassermann reaction, obstructive jaundice nor a miscellaneous group including Weil's disease, amoebic hepatitis, malaria and T.N.T. hepatitis gave a similar fixation with post-arsphenamine livers alone.

As in the sera of cases of post-arsphenamine hepatitis complement-fixing antibodies were not found in every case, so in the livers of cases dying of acute necrosis of the liver during arsenotherapy the specific antigen was not always present. Of 16 livers tested, 10 showed some antigenic activity differing from that of the controls. The titres of complete fixation of these antigens with various sera are shown in Table V. The highest dilution of the liver extract

TABLE V.—*Comparison of the Titres of Various Positive Sera when Tested with Different Active Post-arsphenamine Hepatitis Liver Antigens.*

Antigen.	Highest dilution of antigen giving complete fixation with positive sera.	Highest dilution in which serum gave complete fixation with positive antigens.									
		Σ57.	Σ30.	Σ48.	Σ44.	Σ32.	Σ27.	Σ83.	Σ84.	Σ86.	Σ82*
P-A1	1 : 6	1 : 40	1 : 40	1 : 40	1 : 40	1 : 20	1 : 40	..	..	..	..
P-A2	1 : 3	..	..	1 : 40	1 : 40	1 : 20	1 : 40	..	..	..	..
P-A3	?	1 : 20	1 : 20	..	..	..	..	..	..	..	..
P-A4	1 : 2	1 : 20	1 : 20	..	..	..	..	..	..	..	..
P-A5	1 : 2	1 : 20	1 : 20	..	..	..	..	..	..	..	..
P-A6	1 : 4	1 : 40	1 : 20	..	..	..	..	1 : 5	1 : 5	1 : 5	..
P-A7	1 : 2	1 : 10	..	1 : 20	..	..	..	..	..	..	..
P-A8	2 : 3	1 : 10	..	1 : 40	..	..	..	..	..	..	..
P-A9	1 : 5	..	..	..	..	..	..	1 : 5	..	..	1 : 40
P-A10	1 : 4	..	..	..	..	..	..	1 : 5	1 : 5	1 : 5	1 : 40
Normal	..	0	0	0	0	0	0	0	0	0	0

* This serum gave fixation with unheated antigens only.

which was capable of giving complete fixation with sera containing the specific antibody is also shown in this Table. The titre of the same serum commonly varied with different antigens, while the maximum dilution of the 15 per cent extract showing full activity varied from 2 : 3 to 1 : 6. The last of these positive liver extracts was obtained at a time when the incidence of post-arsphenamine hepatitis was falling.

In only one case (Σ82) was a difference found between the reaction with unheated and that with antigen heated to 56° C. for 30 minutes. In this case complement was fixed only with unheated antigens. The reaction of this serum and the usual type (represented by Σ83) with one antigen treated in various ways are shown in Table VI.

TABLE VI.—*Results of Extracting an Active Liver from Post-arsphenamine Hepatitis in Various Ways.*

Antigen.	Sera.	Serum dilution.	Antigen dilutions. 1 : 2.	1 : 4.	1 : 8.	1 : 16.	1 : 32.	Serum controls.
Simple saline extract	Σ82	1 : 30	++++	++++	++	+	0	0
	Σ83	1 : 5	++++	+++	+	+	0	0
	Arsenotherapy control	1 : 5	0	0	0	0	0	0
Antigen controls	..	..	0	0	0	0	0	..
Phenolized (0·5%) extract	Σ82	1 : 30	++++	++++	++	+	+	
	Σ83	1 : 5	++++	++++	++	+	..	
	Arsenotherapy control	1 : 5	0	0	0	0	0	
Antigen controls	..	..	0	0	0	0	0	
Heated 56° C. 30 minutes and phenolized	Σ82	1 : 30	++	+	+	+	0	
	Σ83	1 : 5	++++	+++	++	+	0	
	Arsenotherapy control	1 : 5	0	0	0	0	0	
Antigen controls	..	..	0	0	0	0	0	

It will be seen that the addition of phenol to a concentration of 0·5 per cent had no effect on the reaction. Heating to 56° C. for 30 minutes and using the supernatant when this extract had been left overnight before centrifuging had no effect on the reaction with Σ83, although no significant fixation remained with Σ82.

Nature of the Reaction.

The attempts which have been made to elucidate the mechanism of the specific reaction between the livers of cases which had died of post-arsphenamine hepatitis and the sera of convalescent and recovered cases of the same disease are listed below. Most of them were based on the theory that the reaction was due to a combination of protein from damaged liver combined with arsenic compounds acting as an antigen. All the artificial antigens listed were made by Dr. M. R. Pollock.

1. Attempts to inhibit the fixation of complement with N.A.B. and mapharside failed. These substances proved anticomplementary when more than 0·25 mg. were present in the system.

2. N.A.B. was diazotized and linked with horse serum, and this was used as an antigen in place of human liver. Tests with this were completely negative.

3. Mapharside was linked to denatured horse serum, by the—SH groups. This substance when injected into rabbits produced specific antibodies. (These results will be reported separately.) Used as an antigen against positive sera from convalescents from post-arsphenamine hepatitis it gave negative results.

4. Rabbit sera which contained specific antibodies against arsenoxide failed to give complement fixation with the positive post-arsphenamine hepatitis liver antigens.

5. Livers from two cases of acute necrosis of the liver which gave negative results in complement-fixation tests with known positive post-arsphenamine hepatitis sera were extracted with saline at + 2° C. for 24 hours and the supernatants then dialysed for 24 hours. The resultant extract gave a faintly positive nitro-prusside reaction. It was treated in four ways:

(*a*) It was used as an antigen in this state.

(*b*) Arsenoxide was mixed with it. The mixture was then dialysed for 24 hours. The resultant extract contained traces of arsenoxide.

(*c*) It was reduced with cyanide. The nitro-prusside reaction then became strongly positive. Arsenoxide was added in excess and the mixture was dialysed for 24 hours. The resultant extract contained arsenoxide and gave a negative nitro-prusside reaction.

(*d*) The extract was heat denatured and then treated as in (*c*).

These extracts were highly anti-complementary, but in the dilutions in which it was possible to use them no positive results were obtained.

6. Presence of arsenic could not be demonstrated in one high titre antigen.

7. A high titre antigen was spun in a Sharples super-centrifuge at a speed and for a time to cause the deposition of a large part of any known virus. After this treatment neither the supernatant nor the deposit showed any antigenic activity. Dr. Markham, who carried out the centrifugation, suggested that this might be due to loss of a very small quantity of material in the walls of the bowl, since the deposit was removed in a cylinder of filter paper placed as a lining around the bowl.

Complement fixation between antigens made from normal and abnormal livers and sera from cases of hepatitis.

As well as the specific fixation between livers and sera from cases of post-arsphenamine hepatitis, a reaction was observed occurring earlier in the disease between sera from certain cases of all three types of hepatitis, and some controls and extracts both from normal livers and those from cases of acute necrosis of liver. This reaction was nearly always more marked in the first specimen of serum obtained than in later specimens and, unlike the reaction found in arsenotherapy hepatitis, was never seen in specimens of serum taken after the recovery of the patient. Complete fixation with serum at a dilution of 1 : 5 or higher was found in nine of 84 cases of infective hepatitis (10·7 per cent), two of 12 cases of homologous serum hepatitis due to known icterogenic serum (16·7 per cent), and ten of 55 cases of post-arsphenamine hepatitis (18·2 per cent). From Table IV it will be seen that in other conditions in which liver damage was present this reaction was found in a similar proportion of cases; while in normals and other cases without known liver damage a positive reaction was rare. The reaction did not bear any close relation to the severity of the

disease, although it was more frequently positive in cases of acute necrosis of the liver than in other cases (Table VII).

TABLE VII.—*Complement-fixation Reaction with Sera from Cases of Acute Necrosis of Liver and Antigens made from Normal Human Livers.*

Case.	Serum dilutions.			
	1 : 5.	1 : 10.	1 : 20.	1 : 40.
Br	++++	++++	++++	++
Wa	++++	++++	+++	++
Wd	++++	+++	Not tested.	
Me	+++	+++	,,	
Sc	++	++	+	+
Ho	++	++	0	0
Go	0	0	0	0
Br	0	0	0	0

This antigen was not destroyed by heating to 56° C. for one hour, but if it was centrifuged immediately after heating, the antigenic activity was found in the deposit. If left overnight before spinning there was no difference in titre between heated and unheated antigen.

This reaction appears to be the same as that described in detail by Eaton, Murphy and Hanford (1944), and the precipitin reaction described by Olitski and Bernkopf (1945), which the authors consider to be similar in causation.

DISCUSSION.

The investigation of the complement fixation between liver extracts and sera from cases of hepatitis has revealed two entirely separate and definite reactions. Olitzki and Bernkopf brought evidence to suggest that the one is the production of an anti-liver antibody occurring in some subjects whose liver has been damaged from any cause. Eaton, Murphy and Hanford, who have described the same reaction in much greater detail, found it to occur in a larger percentage of cases of hepatitis as well as in normal subjects, but had the criteria of fixation which they accepted been used in our work the number of positives would have been increased and the close relation to liver damage would also have been reduced. While the nature of the reaction is clear its significance is not. The reaction does not bear any close relation to the severity of the liver damage, and is probably as common in malaria (this series) and nearly as common in primary atypical pneumonia (Eaton, Murphy and Hanford) as in diseases in which the liver is the organ chiefly damaged.

The other clear-cut reaction is different both in occurring in convalescence instead of in the acute phase of the disease, and in being found only between antigens made from the livers of cases of post-arsphenamine hepatitis and sera from convalescent cases of the same disease. It was at first thought that this reaction was probably due to a combination of arsenic with damaged liver acting as an antigen, and the experiments listed were made in an attempt to establish this theory. Arsenic could not be demonstrated in the antigen, sera containing anti-arsenoxide antibodies failed to react with positive antigens, inhibition experiments using N.A.B. and mapharside as inhibitors failed in any dilution in which the inhibitors alone were not anti-complementary, and none of the many artificial antigens containing in one case N.A.B. and in the others arsenoxide were effective. Therefore this theory must be regarded as unproven.

The other peculiarity of this reaction was that a high percentage of cases gave positive reactions during the period in which post-arsphenamine jaundice was epidemic in military clinics, and that a series of patients gave consistent negatives after the incidence rate fell. This leads back to the possibility that the reaction was in fact associated with an agent, widespread at that time, probably spread by syringe (Salaman, King, Williams and Nicol, 1944), and which has now either disappeared or become relatively rare. This would fit in with Dr. Markham's interpretation of the centrifugation experiment, which could not be repeated because antigens of high titre were not again obtained in sufficiently large a quantity. Further, Beattie and Marshall (1944) have given evidence suggesting that there is no cross-immunity between infective hepatitis and post-arsphenamine hepatitis, and various authors (Beeson *et al.*, 1944 ; Havens, 1945 ; MacCallum and Bauer, 1944 ; MacCallum, 1945, and Paul *et al.*, 1945) have failed to find evidence of cross-immunity between infective hepatitis and homologous serum hepatitis ; although Oliphant (1944) failed to produce hepatitis in 10 volunteers who had previously suffered from homologous serum hepatitis, and were given infective hepatitis material which caused jaundice in four of ten normal volunteers. Most of the evidence suggests a different causation of infective hepatitis from those of post-arsphenamine and homologous serum hepatitis, but there is no evidence either for or against cross-immunity between the latter two conditions.

Some cases of hepatitis during arsenotherapy continued to occur after the alteration of technique which eliminated the likelihood of syringe-spread. This fact, combined with the failure of the complement-fixation reaction in ten successive cases, seven of them tested at all stages of the disease, suggests multiple causation. Such an explanation might account for the high percentage both of convalescent sera and of livers from fatal cases which failed to show activity even during the epidemic period.

Findlay, Martin and Mitchell (1944) report obtaining an antigen by saline extraction of the liver of a fatal case of hepatitis in an African soldier which gave complement fixation with sera from cases of post-vaccinal and infective hepatitis, but not with control sera. The positive sera gave negative fixation with an extract of normal liver. Four other livers were tried, two of which gave some fixation and two others none at all. In the series reported here the liver extract from one case of infective hepatitis gave suggestive results with sera from the same disease, but not with post-arsphenamine hepatitis sera. The remainder of the large series of livers from known or reputed infective hepatitis or homologous serum hepatitis gave negative results.

SUMMARY.

1. A complement-fixation reaction is described occurring between liver extracts from fatal cases and sera from convalescent cases of post-arsphenamine hepatitis among military patients at the time when the incidence of this condition was very high.

2. A complement-fixation reaction between sera of acute cases of all three types of hepatitis and extracts of normal and abnormal livers is reported.

3. Attempts to find a specific complement-fixation reaction in infective hepatitis and homologous serum hepatitis failed.

It is a pleasure to record my thanks to Dr. M. R. Pollock, who made the artificial antigens, to Dr. R. Markham for his help in the centrifugation experiments, and to all those who have so kindly supplied the specimens of liver and serum without which these experiments could not have been made.

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A STUDY OF THE FORMATION, PROPERTIES AND PARTIAL PURIFICATION OF LEUKOTAXINE.

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MENKIN (1936; 1938*a*, *b*, *c*; 1940) showed that a number of inflammatory exudates contain a factor which induces, on intracutaneous injection, a prompt local increase in capillary permeability and a rapid migration of leucocytes through the capillary walls into the area of injection. The liberation of such a substance and its presence in inflammatory exudates offers a reasonable mechanism for the locally increased capillary permeability and leucocyte infiltration observed whenever tissues are injured. Menkin named the factor "leukotaxine," and described a purification process by which he claimed to have obtained it in crystalline form; he concluded that leukotaxine was a polypeptide of comparatively low molecular weight. Menkin also obtained leukotaxine by the tryptic digestion of serum and serum albumin. A further investigation of leukotaxine was undertaken by Duthie and Chain (1939), who obtained substances with the physiological properties of leukotaxine by the action of pepsin, trypsin and papain on a number of proteins; Duthie and Chain were unable to confirm Menkin's claim that leukotaxine was a single crystalline substance, and inclined to the view that there were several polypeptides, formed by the action of proteolytic enzymes on tissue proteins, all having the physiological properties ascribed to leukotaxine.

The work described in the present paper had three main objects. Firstly, we wished to determine whether leukotaxine was involved in the inflammations caused by the well-known war gases, mustard gas, lewisite and phosgene; secondly, we wished to study the general physiological properties of leukotaxine in so far as these had a bearing on the phenomena of inflammation and shock; thirdly, we hoped to be able to purify leukotaxine and obtain some insight into its chemical structure. Unfortunately the work had to be abandoned and our collaboration brought to an end before all these objects were achieved; however, some progress was made towards all of them, and this is now placed on record.

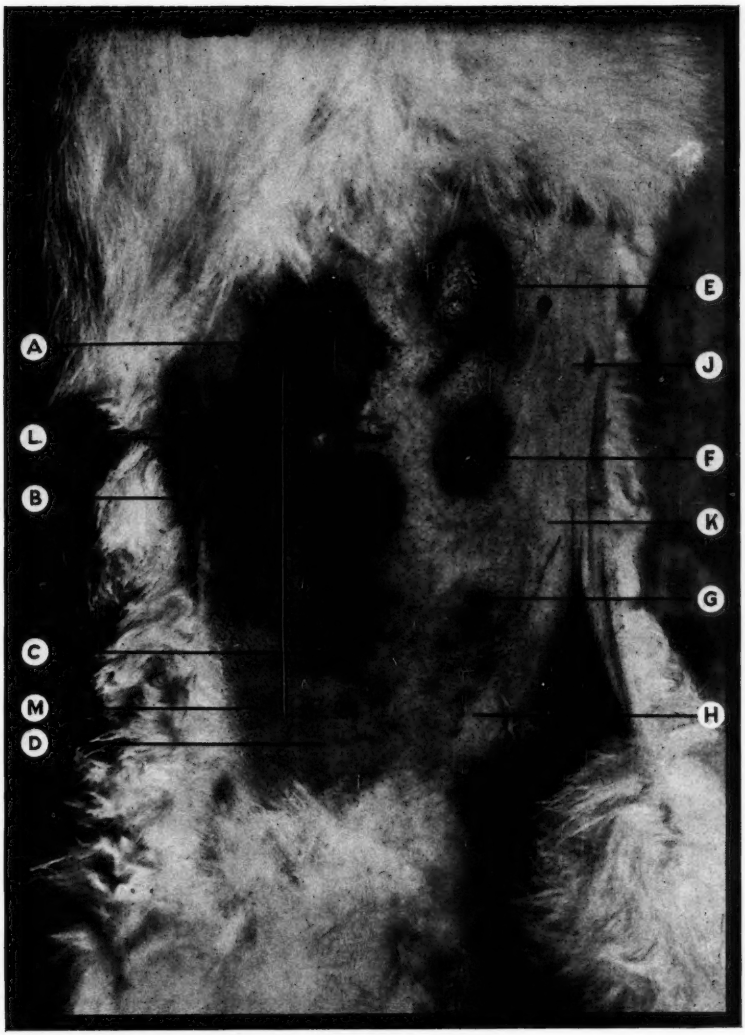
METHOD.

Throughout this work we have used the capillary permeability test as devised by Menkin (1936), and improved by Duthie and Chain (1939). 10 c.c. per kg. body weight of 1 per cent trypan blue or 1·25 c.c. per kg. of 5 per cent pontamine blue in 0·6 per cent saline was injected intravenously into rabbits; immediately afterward 0·2 c.c. of the liquid under test was injected intracutaneously into the previously depilated abdomen of the animal. If the liquid is active a blue colour, due to local leakage of the dye through the capillary walls, appears in 30–60 seconds, and reaches a maximum in 15–20 minutes; the colour change is seen more easily if the skin is swabbed with liquid paraffin. Some typical reactions are shown in Fig. 1.

The test was made roughly quantitative by injecting successive dilutions of the solution under test until a dilution was reached at which no colour change was visible; contrary to the findings of Duthie and Chain, we found that the tonicity or pH of the injected fluid had little or no effect on assays carried out in this way. The results are expressed in terms of "permeability units"; one "unit," when present in 0·2 c.c. of solution, produces a just visible colour reaction on injection. Owing to considerable variation in the capillary response from day to day and from rabbit to rabbit it was found necessary to standardize the assay by reference to the activity of standard samples prepared from material once purified by the charcoal-pyridine process (see below); the standards were stored in a desiccator and made up in solution as required, each new experimental sample being compared directly, using the same rabbit, with the standard. Several batches of the standard have been used, each having been first assayed against earlier standards; the standards themselves, however, show some loss of activity on prolonged keeping, and for this reason our "units" have little absolute quantitative significance; nevertheless the activities quoted in any one section of the paper give a fair estimate of the relative activities of the samples under discussion.

FIG. 1.—Abdomen of rabbit showing reactions to various fluids. 0·2 c.c. injected at each site. 10 c.c. of 1 per cent trypan blue injected intravenously. The effect on capillary permeability is demonstrated by the accumulation of dye in the various skin areas.

Key.—A. Goat pleural exudate; undiluted. B. Ditto; diluted 1 : 10. C. Ditto; diluted 1 : 100. D. Ditto; diluted 1 : 200. E. Mustard gas blister fluid (human); undiluted. F. Ditto; diluted 1 : 5. G. Ditto; diluted 1 : 10. H. Ditto; diluted 1 : 20. J. Histamine acid phosphate; 1 per cent in isotonic saline. K. Isotonic saline. L. Goat phosgene lung-oedema fluid; undiluted. M. Goat phosgene lung-oedema fluid; diluted 1 : 2.



Cullumbine and Rydon.

PRESENCE OF LEUKOTAXINE IN PATHOLOGICAL EXUDATES.

Tested in the above manner, the blister fluids collected from burns produced thermally and by the application of mustard gas, lewisite and bis-(β-chloroethyl)-methylamine had a fairly high activity in producing increased capillary permeability, while the oedema fluid obtained from the lungs, bronchi and pleura of animals gassed with phosgene had a smaller, but still definite, activity. The chemical burns were produced by the application of 2 mm. diameter drops of the liquid vesicants to the forearms of human subjects; 24 hours later the fluid was removed aseptically from the resulting blisters. Thermal burns were produced by the application of a tube of water at 80° for 5 minutes to the skin of rabbits under nembutal anaesthesia. To obtain the phosgene lung-oedema fluids, goats were exposed to phosgene (475 mg./m³) for 30 minutes; immediately after death, which occurred in 6–18 hours, the chest cavity of each goat was opened, and the fluids found in the pleural cavity draining from the bronchi and expressed manually from the oedematous lungs were collected separately.

The active factors in all these exudates, and in the inflammatory exudate produced in goats by Menkin's method (intrapleural injection of turpentine; see p. 4), were precipitated from the cell-free (centrifuged) exudates by half-saturation with ammonium sulphate, but not by the addition of one volume of pyridine and two of acetone, and diffused readily through collodion or cellophane membranes. It seemed probable, therefore, that all the exudates contained Menkin's leukotaxine or some substance very similar to it in chemical and physical properties.

Local increased capillary permeability occurs early in the chain of biochemical processes leading up to vesication; this has been demonstrated by experiments on rabbits and on human subjects. The experiments on rabbits, prepared as for the assay of leukotaxine, are recorded in Table I. A rather

TABLE I.—*Early Occurrence of Increased Capillary Permeability in the Development of Vesicant Lesions in Rabbits.*

Vesicant.	Contamination (mm. diameter drop).	Time for first appearance of signs of increased capillary permeability.
Mustard gas	0·5	3 minutes
	1·1	3 ,,
	2·0	3 ,,
Lewisite	0·5	2 ,,
	1·1	1 minute
	2·0	20 seconds

On the skin of a rabbit a 1·1 mm. diameter drop of lewisite produces a gross and spreading subcutaneous oedema evident within 5–6 minutes, and central necrosis within 15 minutes. A similar drop of mustard gas produces, within 2–5 minutes, only a pallor of the skin with a slight localized subcutaneous oedema.

different technique had necessarily to be used with the human subjects; mustard gas (1·1 mm. diameter drop) was applied to the forearm at various times before the intravenous injection of Evans blue, and the time interval elapsing before the appearance of a blue stain beneath the skin at the site of application of the mustard gas was noted; the results are given in Table II. It will be noted

TABLE II.—*Early Occurrence of Increased Capillary Permeability in the Development of Mustard Gas Lesions in Man.*

Subject.	Dosage of Evans blue (mg./kg.).	Time between application of mustard gas and injection of dye (mins.).	Result.
1	0·2	60	Rapidly and obviously blue.
		30	
		5	Blue 20 minutes after injection.
2	0·4	80	Rapidly and obviously blue.
		40	
		20	
3 and 4	0·8	30	Rapidly and obviously blue.
		20	
		10	

that, in man, there is evidence of local increased capillary permeability within 10 minutes of the application of a 1·1 mm. diameter drop of mustard gas to the skin; the earliest clinical sign produced by such a drop of mustard gas under the same conditions of temperature and humidity is the commencement of erythema 60–120 minutes after application. In one of the subjects (No. 2) intradermal injection of 0·2 c.c. of a solution of leukotaxine (100 units/c.c.) prepared from goat inflammatory exudate produced a local blue area around the site of injection, as did a similar injection of 0·2 c.c. of mustard gas blister fluid.

If this early occurrence of local increased capillary permeability is due to the local formation or liberation of leukotaxine and not to some direct physical effect of the vesicant, it should be possible to extract leukotaxine from the developing lesion; this has been done. Of several extraction techniques tried, the following proved the most satisfactory: Mustard gas (2 mm. diameter drop) or lewisite (1·1 mm. diameter drop) was placed on the depilated skin of a rabbit's abdomen; the contaminated area of skin was excised after various intervals. The excised skin (*ca.* 2 g.) was minced under 10 c.c. of phosphate buffered normal saline (pH 7·4), ground in Phillips' tubes and shaken for 30 minutes; the supernatant fluid obtained on centrifuging was then assayed for leukotaxine. Similar extractions were carried out with skin burnt thermally by the application under nembutal anaesthesia of a tube of hot water (80°) for 5 minutes. The results are given in Table III, each value being the average of

TABLE III.—*Extraction of Leukotaxine from Developing Skin Burns.*

Time after contamination or burning (mins.).	Yield of leukotaxine (units/g. of skin).		
	Mustard gas.	Lewisite.	Thermal burn.
30	50	250	150
60	200	250	120
120	500	500	500
240	1,000	500	500

twelve assays on different pieces of skin from different rabbits; control extractions were carried out with normal skin, which gave an average yield of 40 units/g. It will be seen that there is good evidence for the rapid and progressive liberation of leukotaxine in the developing burn.

FORMATION OF LEUKOTAXINE BY ENZYMIC HYDROLYSIS OF PROTEINS.

The previous workers obtained leukotaxine by the enzymic hydrolysis of a number of proteins; we have confirmed and extended these observations. The protein under investigation (200 mg.) in 10 c.c. of a suitable buffer (pH 2 for pepsin; pH 7·8 for trypsin) containing 20 mg. of the appropriate enzyme was kept at 37° for various times; the solution was then neutralized if necessary, centrifuged and assayed in the usual way. The results are given in Table IV. It is evident that leukotaxine is readily formed by the action of pepsin and trypsin on blood fibrin and blood albumin, less readily from casein, zein and myosin, and hardly at all from gelatin, insulin and silk fibroin. Although we do not place undue reliance on such experiments, the results may perhaps indicate that tryptophan (absent from gelatin, insulin and silk fibroin) or some derivative of tryptophan is an essential component of leukotaxine.

Beloff and Peters (1945) have isolated from skin a proteinase which may well be involved in the development of thermal burns. It was clearly desirable to determine whether this skin proteinase resembled pepsin and trypsin in being able to form leukotaxine from proteins, and we were enabled to investigate this point with a specimen of the skin proteinase kindly given to us by Professor R. A. Peters, F.R.S. The dried rat-skin preparation supplied was extracted for 30 minutes with 5 c.c. of M/15 phosphate buffer, pH 7, containing 250 mg. of added potassium chloride. The centrifuged extract was used at once for the following experiments; in each case the mixtures were kept at 37° for an hour, centrifuged and assayed. In Series I the proteinase was extracted from the dried skin at room temperature, and in Series II at 37°. The results were as follows:

	Reaction mixture.	Activity of product (units/c.c.).
SERIES I:		
Blank.	1 c.c. proteinase + 1 c.c. water	10
Fibrin.	1 c.c. proteinase + 1 c.c. water + 10 mg. blood fibrin	100
Casein.	1 c.c. proteinase + 1 c.c. water + 10 mg. casein	5
Blank.	2 c.c. water + 10 mg. blood fibrin	5
SERIES II:		
Blank.	1 c.c. proteinase + 1 c.c. water	0
Fibrin.	1 c.c. proteinase + 1 c.c. water + 10 mg. blood fibrin	80
Albumin.	1 c.c. proteinase + 1 c.c. water + 10 mg. blood albumin	0
Blank.	2 c.c. water + 10 mg. blood albumin	2

The undiluted proteinase on immediate injection had an activity of 5 units/c.c. It is clear from these results that the skin proteinase is active in producing

leukotaxine from blood fibrin. The negative result with blood albumin is probably to be ascribed mainly to the pH (7·0) of the reaction mixture since a similar, but less marked, difference between blood fibrin and blood albumin was observed with trypsin, acting at pH 7·8, but not with pepsin, acting at pH 2 (see Table IV).

TABLE IV.—*Formation of Leukotaxine by the Action of Enzymes on Proteins.*

Enzyme.	Substrate.	Activity (units/c.c.) after incubation for—					
		30 mins.	1 hr.	3 hrs.	5 hrs.	6 hrs.	20 hrs.
Pepsin	Blood fibrin	1500	4000	3000	1500	500	—
	Blood albumin	—	—	—	—	500	—
	Casein	—	—	—	—	75	—
	Gelatin	—	—	—	—	2·5	—
	Rabbit myosin	—	—	—	—	—	100
	Zein	—	—	—	—	—	100
	Insulin	—	—	—	—	—	20
	Silk fibroin*	—	—	—	—	—	20
Trypsin	Blood fibrin	—	—	—	—	2400	—
	Blood albumin	—	—	—	—	400	—
	Casein	—	—	—	—	400	—
	Blank	—	—	—	—	80	—

* Silk fibroin pre-treated with cuprammonium hydroxide (Bergmann and Niemann, 1938).

Clearly the action of the skin proteinase on tissue proteins may be responsible for the liberation of leukotaxine in the early stages of vesication. Whether this is brought about by the liberation of the proteinase by some direct action of the vesicant on the tissues, or by the destruction or inactivation by the vesicant of some inhibitor normally present, thus rendering the skin proteinase capable of acting, is not clear; the latter alternative is to some extent favoured by the observation that intracutaneous injection of the skin proteinase itself does not cause the rapid liberation of leukotaxine, since there is no significant local increase of capillary permeability.

Formation of Leukotaxine by the Action of Mustard Gas on Serum.

Fresh horse serum was gently stirred at 37° over a layer of mustard gas; samples were pipetted off at intervals, kept at room temperature for an hour and at 0° overnight to complete the hydrolysis of dissolved mustard gas, and then assayed for leukotaxine. The supernatant liquid had an activity of 20 units/c.c. after stirring for 30 minutes (when the pH was still 7·5), 60 minutes pH 6·5) and 120 minutes (pH 5·0); the activity of the 30-minute product remained unchanged on incubation, in the absence of mustard gas, for periods up to 48 hours. No capillary permeability activity was produced when horse serum was incubated alone for 24 hours. It is clear from these experiments that not only proteolytic enzymes but also the simple chemical reagent, mustard gas, will liberate leukotaxine from serum proteins.

PHYSIOLOGICAL PROPERTIES OF LEUKOTAXINE.

Chemotactic Properties.

Menkin's leukotaxine had the property of inducing, in cutaneous tissue, migration of polymorphonuclear cells through the endothelial walls of small blood vessels. Our preparations, both crude and purified, also possess this chemotactic property.

Turpentine (1 c.c.) was injected into the peritoneal cavity of a rabbit; 18 hours later glass capillary tubes, sealed at one end and containing saline solutions of crude leukotaxine, leukotaxine purified by the charcoal-pyridine method, and histamine were inserted into the peritoneal cavity during nembutal anaesthesia. Six hours later the tubes were recovered and examined; those containing the crude and purified leukotaxine were swarming with leucocytes, whereas those containing histamine contained markedly fewer cells. In another type of experiment, saline solutions of leukotaxine were injected into the skin of rabbits and guinea-pigs. The animals were sacrificed 30–60 minutes later and sections of the skin were made. In the sections taken 30 minutes after the injection of leukotaxine the lumina of the small blood vessels were crowded with polymorphonuclear leucocytes; after 60 minutes these had migrated into the extracapillary cellular spaces. Saline solutions of histamine had no such effect.

Menkin (1940) claimed to have observed the clustering of supravitally stained polymorphonuclear leucocytes around solid particles of leukotaxine after incubation on a slide; we were quite unable to observe this effect owing to the rapid solution, and diffusion over the whole field, of our leukotaxine. The solubility of our product likewise prevented the direct observation of its chemotactic effects on implantation into bone-marrow tissue cultures.

Distinction from Histamine.

Menkin (1940) has listed a number of distinctions between leukotaxine and histamine; we have carried out a number of other experiments which lead to the conclusion that leukotaxine is different from histamine. In the first place histamine, unlike leukotaxine, is not chemotactic for leucocytes. The effects of leukotaxine and histamine on local capillary permeability after intracutaneous injection are quite different; in rabbits prepared as for the assay of leukotaxine (p. 1) intracutaneous injection of leukotaxine produces a diffuse blue area, whereas histamine is either without effect (see Fig. 1), or gives a narrow blue ring with a central unstained area. Unlike histamine, leukotaxine does not cause contraction of isolated guinea-pig intestine; a quantitative experiment showed that 6 mg. (550 units) of once-purified leukotaxine contained less than 2 μg. of histamine. Isolated guinea-pig intestine does not become refractory after treatment with leukotaxine, histamine still causing the contraction of the tissue so treated. Clearly the claims of Bier and e Silva (1938) to have shown that leukotaxine is identical with histamine cannot be upheld.

Destruction of Leukotaxine by Serum.

It might be suggested that the local production of leukotaxine at the site of a burn and its subsequent absorption into the general circulation was a factor in the production of the "shock" syndrome. However, no untoward symptoms are produced by the intravenous injection of leukotaxine in rabbits, and Menkin (1940) has shown that intravenous injection of leukotaxine does not affect the blood pressure of cats. Accordingly it seemed possible that leukotaxine was destroyed or inactivated in the blood, and we carried out a number of experiments to test this point. Leukotaxine was dissolved in rabbit serum, incubated for various times at 37° and then assayed as usual; control experiments were carried out with rabbit serum alone and with solutions of leukotaxine in buffered

saline. In these experiments it was found necessary to work with sterile solutions, since otherwise the leukotaxine lost its activity rapidly even in buffered saline. The results are given in Table V, from which it will be seen that leukotaxine is

TABLE V.—*Activity of Solutions of Leukotaxine after Incubation at* 37°.

Period of incubation.	Activity (units/c.c.) of solutions in—				
	Buffered saline.*	Rabbit serum.*	Rabbit plasma.*	Horse serum.*	Buffered saline.†
0	100	100	100	100	100
3 minutes . .	100	25	—	—	—
10 ,, . .	100	5	—	—	—
30 ,, . .	100	5	—	—	—
40 ,, . .	100	5	—	—	—
4½ hours . .	100	5	—	—	70
18 ,, . .	100	5	10	20	40
24 ,, . .	100	0	0	—	25

* Sterile materials. † Unsterilized.

Sterile rabbit serum, rabbit plasma and horse serum, assayed as controls, gave negative results. All the assays are the mean of 8 experiments.

rapidly destroyed, or inactivated, at 37° in the presence of normal plasma or serum; the action is probably enzymic, since no destruction of leukotaxine occurred on incubation for 4 hours with solutions of precipitated rabbit serum euglobulin, pseudoglobulin or albumin.

If, however, leukotaxine is incubated with serum from rabbits in which a state of "shock" has been produced, e.g. by the injection of lewisite oxide subcutaneously, by the application of mustard gas to the skin or by thermal burning, then no inactivation takes place; this is illustrated in Table VI.

TABLE VI.—*Activity of Solutions of Leukotaxine after Incubation with Serum from Rabbits Suffering from Shock.*

Treatment of rabbit.	Activity of solution (units/c.c.) after incubating with serum at 37° for—				
	0.	1 hr.	4 hrs.	24 hrs.	48 hrs.
None	400	250	50	25	0
2 mg./kg. lewisite oxide injected 24 hours before bleeding .	550	500	450	600	500
2 mg./kg. lewisite oxide injected 48 hours before bleeding .	450	400	300	250	300
20 mg./kg. mustard gas applied to skin 24 hours before bleeding .	400	500	550	600	450
Thermal burn on abdomen 24 hours before bleeding . .	500	400	450	600	350

All figures are the mean of 3 rabbits.

Capillary Leakage of Protein under the Influence of Leukotaxine.

As an indication of the degree of enhanced capillary permeability induced by leukotaxine, 10 c.c. per kg. body weight of a solution of blue dyed serum albumin

(4·5 per cent protein), kindly presented to us by Dr. J. F. Danielli, was injected intravenously into rabbits and followed by intracutaneous injection of leukotaxine into the previously depilated abdomen. Local blue areas were produced, indicating that the increase of capillary permeability was sufficiently great to permit even protein molecules to diffuse through the altered capillary walls.

PREPARATION AND PURIFICATION OF LEUKOTAXINE.

Sources.

Blister fluids from chemical or thermal burns are not convenient preparative sources of leukotaxine since they can only be obtained in comparatively small amount. In our earlier experiments we employed as sources either the inflammatory exudate produced by intrapleural injection of turpentine in goats (*cf.* Menkin, 1940), or peptic digests of blood fibrin (*cf.* Duthie and Chain, 1939). With a view to confirming the supposed polypeptide nature of leukotaxine we attempted to carry out, at the suggestion of Professor C. Lovatt Evans, F.R.S., some experiments on its destruction by the proteolytic enzymes of *succus entericus*. To our surprise we found that *succus entericus* is itself extremely rich in leukotaxine, presumably formed by the action of its proteolytic enzymes on the tissue proteins during the process of preparation; control experiments showed that *succus entericus* contained only small amounts of histamine quite insufficient to account for its activity in increasing local capillary permeability. In our later work we frequently used *succus entericus* as a preparative source of leukotaxine. All three sources of leukotaxine mentioned in the present paragraph can be worked up by the methods described below; in practice the richest and most readily available sources have proved to be rabbit *succus entericus* and the peptic digest of blood fibrin.

Preparation of Crude Leukotaxine.

It should be mentioned at once that many of the solvents (especially pyridine, acetone and alcohol) used by Menkin in his purification processes themselves give marked positive reactions in the capillary permeability test, a fact which Menkin does not seem to have realized. In view of this, great care was taken throughout our work to ensure the absence of such substances from any preparations to be tested.

(a) *From turpentine inflammatory exudate.*

Turpentine (1·5 c.c.) was injected intrapleurally into each of three goats. The goats were killed 24 hours later, and the pleural exudate (600 c.c.) collected and at once treated with pyridine (600 c.c.) and acetone (1200 c.c.). The precipitated protein, centrifuged off as soon as possible, proved to be inactive in the capillary permeability test after re-solution in water. Evaporation of the supernatant fluid under reduced pressure, followed by drying for 24 hours in a vacuum desiccator over sulphuric acid, gave crude leukotaxine (9 g.) as a dark brown, hygroscopic, semi-solid mass with an activity of about 4 units/mg. Application of this isolation procedure to reconstituted human plasma and dog serum incubated at 37° for 24 hours with their own volume of pyridine gave only inactive products.

(b) *From peptic digest of blood fibrin.*

A suspension of blood fibrin (5 g.) in water (100 c.c.) was brought to pH 2 by the addition of 10 *N*-HCl (10 c.c.). Pepsin (B.D.H., 500 mg.), dissolved in a little 0·1 *N*-HCl, was added and the mixture kept at 37° for an hour ; 2 *N*-NaOH was added to bring the solution to pH 8. The resulting solution (160 c.c.) was then worked up as described for the turpentine inflammatory exudate. The product, 6·32 g., had an activity of 62 units/mg.

(c) *From* succus entericus.

Succus entericus from rabbits, prepared in the usual way, was diluted with a little water and then subjected to the extraction procedure described for the turpentine inflammatory exudate. The product had an activity of 37 units/mg.

Purification of Crude Leukotaxine by Adsorption.

The further stages of Menkin's purification process did not seem to us to be very satisfactory, and there is little in Menkin's writings to show that he did indeed achieve any considerable degree of purification ; adsorption methods (*cf.* Duthie and Chain, 1939) seemed more promising and we turned our attention to them. Crude leukotaxine, prepared from turpentine inflammatory exudate, was adsorbed from aqueous solution by charcoal, fuller's earth, heavy magnesium carbonate and calcium carbonate ; it was not adsorbed by activated alumina, while variable results were obtained with kieselguhr. Further investigation showed that the leukotaxine could be eluted from magnesium carbonate with 5 per cent pyridine and from charcoal with 20 per cent pyridine ; the latter procedure was used for large-scale working. Leukotaxine from other sources (peptic blood fibrin, *succus entericus*, serum incubated with mustard gas) had precisely similar adsorption properties ; this may be taken to indicate that the active factors from all these sources are, if not identical, at least chemically very similar.

The procedure finally found satisfactory is as follows. Crude leukotaxine is dissolved in 10 parts of water and shaken for 10 minutes with 1 part of activated charcoal (B.D.H.). The charcoal is centrifuged off and the supernatant, which contains most of the inorganic matter and is usually inactive, discarded. The charcoal is shaken for 5 minutes with 10 volumes of 5 per cent pyridine and again centrifuged. The process is repeated with 20 per cent pyridine, and the charcoal is finally shaken for at least 3 hours with 20 per cent pyridine ; this final prolonged shaking is usually necessary to ensure the maximum recovery of active material. All three pyridine extracts are assayed after evaporation to dryness *in vacuo*. The final 20 per cent pyridine extract usually gives the most active material, but the results are somewhat variable ; the first 20 per cent pyridine extract may contain much, sometimes most, of the activity, and even the 5 per cent pyridine extract may be appreciably active. The active extracts are evaporated to dryness *in vacuo*, finally in a vacuum desiccator over sulphuric acid ; they may be stored in a desiccator at room temperature. Their keeping properties are rather variable ; considerable loss of activity is often observed during the first week or two, after which they lose activity only comparatively slowly. For this reason " standards " were always prepared from aged specimens. The stability of various preparations on long storage (4–12 months) decreases in the order inflammatory exudate > *succus entericus* > peptic fibrin.

The average yields and activity of leukotaxine preparations from various sources are given in Table VII.

TABLE VII.—*Yield and Activity of Leukotaxine from Various Sources.*

Source.	Crude product.		Product purified by charcoal-pyridine.	
	Yield (mg.).	Activity (units/mg.).	Yield (mg.).	Activity (units/mg.).
Rabbit *succus entericus* (average for 1 rabbit)	700	31	100–150	125
Goat *succus entericus* (3 goats)	1,300	29	250	143
Peptic digest of blood fibrin (5 g.)	6,300	62	524	500 (unstable)
Goat pleural exudate (turpentine) (1000 c.c. from 6 goats)	8,700	19	230	167
Mustard gas blister fluid (human) (5 c.c.)	132	1·2	6	6·7
Phosgene lung-oedema fluid (3 goats)	14,400	3·5	370	25

Further Purification of Leukotaxine by Fractional Dialysis.

Unless the once purified material is grossly contaminated with inorganic matter (e.g. in working up peptic digests) or is very inactive, it is not usually profitable to carry out a second purification by the charcoal-pyridine method. Preliminary experiments on the use of other adsorbents for further purification were discouraging, and we therefore turned our attention to fractional dialysis, since it was known that leukotaxine passed fairly readily through cellophane membranes.

The material to be purified (about 50 mg. in 1 c.c. of solvent) was contained in a cellophane bag rotated at 150 r.p.m. in a suitable solvent; from time to time the outer solution was removed for test and replaced by fresh solvent. In our initial experiments water was used as solvent in both inner and outer vessels. The early dialysates were highly active, indicating that leukotaxine diffused rapidly and therefore had a comparatively low molecular weight. However, the total recovery of active material in these experiments was very low and the inner solution became opalescent and acid in reaction; it was thought that this loss of activity might be due to adsorption of leukotaxine on the protein precipitated as the solution became acid, and this view was confirmed experimentally. On acidifying a solution of leukotaxine containing some protein a precipitate was produced; after centrifuging, the supernatant, brought to pH 8, was inactive, as was the precipitate when re-dissolved at pH 8. Clearly, not only is leukotaxine adsorbed on protein precipitates in acid solution, but it is also irreversibly destroyed in the process.

The difficulty was avoided by working in alkaline solution, using 5 per cent pyridine as the solvent in both inner and outer vessels. The results varied slightly from experiment to experiment; the first dialysate, collected during the first 15 minutes, contains most of the inorganic material and usually yields a rather inactive product on evaporation. Subsequent dialysates, up to about 1 hour, usually yield the most active products. Details of a typical experiment follow.

Once purified leukotaxine (39 mg.; 77 units/mg.) from rabbit *succus entericus* was dissolved in 1 c.c. of 5 per cent pyridine and placed in a small cellophane bag rotated (150 r.p.m.) in 5 c.c. of 5 per cent pyridine. From time to time the

outer liquid was replaced and the successive dialysates evaporated and assayed, with the following results:

Dialysate	Product. Weight (mg.).	Activity. Total (units).	Activity. Units/mg.
0–15 minutes	13	500	38·5
15–30 ,,	4	500	125
30–45 ,,	4	1000	250
45–60 ,,	3	1000	333
1–2 hours	3	100	33
2–6 ,,	8	0	0
Residue	6	500	83
Total	41 mg. (105%)	3600 units (120%)	

This method of purification removes salts, proteins and any colloidal charcoal and gives very clean products. On the average, applied to material once purified by the charcoal-pyridine method, it gives products of about doubled activity in about 30 per cent yield.

CHEMICAL PROPERTIES OF LEUKOTAXINE.

Purified leukotaxine is a pale yellow, non-crystalline, hygroscopic mass which is readily soluble in water; it is notably heat-stable, autoclaving at 120° for 30 minutes causing no serious loss of activity. We have repeatedly obtained preparations with an activity of 300–500 units/mg., i.e. materials which produce an observable response at a dilution of 1/60,000 to 1/100,000. Such preparations are 6–10 times as active as those described by Duthie and Chain (1939); notwithstanding this comparatively high activity we were not satisfied that these materials were sufficiently pure to warrant any quantitative chemical investigation, and our work had, unfortunately, to be abandoned before we could make any further progress with the purification.

Our chemical evidence on the nature of leukotaxine is therefore based solely on colour reactions. Purified samples always gave positive ninhydrin and positive, but usually very feeble, biuret reactions, while the Molisch reaction was negative; this suggests that the products were polypeptides of comparatively low molecular weight. Purified samples invariably gave positive reactions for tryptophan (Cole test) and tyrosine (Millon and Totani reactions); it is thus possible that these two amino-acids are essential components of leukotaxine, a conclusion in agreement so far as tryptophan is concerned with the enzymic experiments quoted on p. 37. We do not, however, put forward these conclusions without reserve, and can only observe that all our results are in agreement with the view that leukotaxine, prepared from a wide variety of sources, is either a single polypeptide of comparatively low molecular weight, or a series of very similar compounds of this same general type.

DISCUSSION.

It is clear from our results that leukotaxine is a common factor in various types of inflammatory process, e.g. thermal burns, vesicant burns, lung oedema

produced by phosgene. Its early formation provides a satisfactory explanation of the increased capillary permeability which must precede the development of a vesicle and the production of lung oedema; in the case of vesication some ancillary mechanism resulting in the weakening of cutaneous tissue must also be involved. We have no certain evidence that the active factors from all these inflammatory processes are identical, but the chemical evidence does suggest that they are at least very similar; the term "leukotaxine" as used in this paper may, therefore, denote either a single substance or a group of several closely related substances with the common property of inducing a local increase of capillary permeability.

The relation of leukotaxine production to the phenomena of "shock," especially shock produced by vesicants, is not altogether clear. On the one hand leukotaxine is not destroyed or inhibited by the sera of animals in a state of shock, whereas it is destroyed by the sera of normal animals; on the other hand leukotaxine is completely absent from the sera of "shocked" animals. Further investigation may resolve these difficulties, but our own work had to be terminated before we could make any useful contribution to the problem.

Our results suggest several ways in which leukotaxine may be produced in various types of inflammatory process. In chemical burns some may be formed by the direct action of the vesicant, since we have shown that leukotaxine is produced by the action of mustard gas on serum. However, a more general mechanism would seem to be its production from tissue proteins by the action of a proteinase (similar to or identical with the skin proteinase of Beloff and Peters, 1945), either liberated from the tissues under the influence of heat or chemical agents, or enabled to act by the inhibition, by these agencies, of some anti-enzyme normally present in the tissues.

SUMMARY.

1. A substance, or substances, similar to Menkin's leukotaxine, has been demonstrated in blister fluids produced thermally or by the action of the vesicants mustard gas and lewisite, and in phosgene lung oedema fluid. The rate of production of leukotaxine in thermal and chemical burns has been studied.

2. The production of leukotaxine from a number of proteins (but not from gelatin, insulin and silk fibroin) by pepsin and trypsin and the skin proteinase of Beloff and Peters has been demonstrated.

3. It has been shown that leukotaxine is produced by the direct action of mustard gas on serum.

4. Evidence is presented that leukotaxine is not identical with histamine; its leucotactic properties have been confirmed.

5. Leukotaxine is rapidly destroyed or inactivated by normal plasma or serum, but not by serum from animals suffering from "shock."

6. A method of preparation and partial purification of leukotaxine is described; the best preparative sources are *succus entericus* and a peptic digest of blood fibrin.

7. Leukotaxine may be a polypeptide of comparatively low molecular weight containing tryptophan as an essential component.

8. The role of leukotaxine in vesication and in the genesis of "shock" is discussed.

We are indebted to the Chief Scientific Officer, Ministry of Supply, for permission to publish this paper, and to numerous colleagues, at Porton and elsewhere, for stimulating discussions.

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FURTHER OBSERVATIONS ON THE CHEMOTHERAPY OF EXPERIMENTAL GAS GANGRENE: FLAVAZOLE, MARFANIL, V187 AND V335.

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RECENT publications have shown that the mortality rate in clostridial gas gangrene is still high, in spite of the use of powerful therapeutic agents and improved surgical technique. There is, however, the reassuring finding of MacLennan and Macfarlane (1944) that in the Sicilian and Italian campaign the mortality rate fell from 70 to 30 per cent coincident with an improvement in the general standard of treatment, and that the mortality rate was lowest among those who received adequate surgical and antitoxin treatment within 6 hours of diagnosis. There have also been very favourable reports of the value of treatment with penicillin, but we and others have observed fatalities even in cases where complete sterilization of the clostridial infection was attained, and in spite of adequate antitoxin dosage. As has been suggested recently by MacLennan and Macfarlane (1945*a*, *b*), these deaths may have been due, not to the direct effects of the clostridial infection, but to some toxic substance derived from the damaged muscle.

It is thus evident that the treatment of established gas gangrene is still a major problem, and in view of the fact that the incidence of gas gangrene has been between 0·3 and 1·0 per cent of all United Kingdom and Empire troops wounded in the different campaigns of this war, it is all the more important that efficient prophylactic measures should be employed. The great success of tetanus toxoid in abolishing tetanus has suggested the use of prophylactic injection of toxoid prepared from the clostridia of gas gangrene. However, difficulties have been encountered in the large-scale preparation of potent toxoid, so that this method of protection is still in the experimental stage. We therefore

consider that an essential prophylactic measure is the immediate treatment of all wounds with an antiseptic capable of preventing the development of gas gangrene infection, in addition to the removal of all devitalized tissues which might promote the growth of bacteria.

With the aim of finding drugs suitable for this purpose we have investigated the therapeutic action of a large number of substances in experimental gas gangrene infections in mice. The mouse is perhaps not the most suitable animal for the experimental study of gas gangrene infection, in that it requires a very much larger number of organisms to produce a fatal infection in the mouse than in the guinea-pig. However, by the choice of suitable strains of clostridia we have been able to obtain consistent results by intramuscular injection over a number of years. Uncertainties which might arise from variations in the amount of preformed toxin in the inocula have been avoided by using washed organisms suspended in a solution of calcium chloride. This method has the further advantage that it produces an overwhelming bacterial invasion of the tissues at an early stage of the infection, and thus provides a highly suitable test for therapeutic substances.

In a previous communication we have shown that highly active substances against *Cl. welchii* and *Cl. septicum* infections are penicillin and sulphathiazole respectively, whereas *Cl. oedematiens* infection is less responsive to treatment (McIntosh and Selbie, 1943). On the basis of our findings we have recommended a powder mixture containing 1 part of proflavine and 99 parts of sulphathiazole as a prophylactic application to wounds (McIntosh, Selbie, Hudson, Patey, Parkes, McMullen and Pile, 1944). Since then a further series of drugs has been tested, and an account of the results obtained with the more active of these will be given here.

EXPERIMENTAL.

The Drugs Tested.

The drugs which have proved active in this series of tests are four new acridine compounds, two sulphone compounds, and marfanil.

Acridine compounds.—Two of the new acridine compounds, nitracrin (5-amino-2-nitro-7-ethoxyacridine, Albert and Gledhill, 1942) and suproflavine (2 : 8-diamino-1 : 9-dimethylacridine, Albert, Rubbo, Goldacre, Davey and Stone, 1945) were supplied by Dr. A. Albert of Sydney University. The two others, which have been prepared by the Research Laboratories of Boots, Ltd., are acridine-sulphathiazole compounds, flavazole (2 : 8-diaminoacridine-sulphathiazole or proflavine-sulphathiazole) and 5-aminoacridine-sulphathiazole. An account of the favourable results obtained with flavazole in the treatment of infected wounds has already been published (McIntosh, Robinson, Selbie, Reidy, Blake and Guttmann, 1945).

Sulphones.—The two sulphones are V187, or *p*-methylsulphonylbenzamidine hydrochloride, and V335, or *p*-methylsulphonylbenzylamine hydrochloride, both synthesized by Evans, Fuller and Walker (1944) in the course of work on the chemotherapy of typhus. These authors have shown that V187 and V335 are similar to the related compound marfanil in their therapeutic activity against *Cl. welchii* infection in guinea-pigs, and that V187 and marfanil are equally active against *Cl. septicum* and *Cl. oedematiens*.

Marfanil.—Marfanil, or *p*-sulphonamidobenzylamine hydrochloride (Miller, Sprague, Kissinger and McBurney, 1940; Klarer, 1941) has been shown by Domagk (1942), Evans, Fuller and Walker (1944), and Hamre, Walker, Dunham, van Dyke and Rake (1944) to have considerable activity against experimental anaerobic infections, and has been used prophylactically by the German army.

Bacteriostatic properties.

Bacteriostatic tests of these drugs have been made in an infusion meat extract with 1 per cent Witte peptone, which does not inhibit sulphonamide activity. Several strains of each of a number of aerobic and anaerobic organisms were sown in this medium in the presence of two-fold dilutions of the drugs, and the results were read after incubation for 24 hours.

The results of these tests are shown in Table I, where it will be noted that sulphathiazole is exceptional in this series of drugs in that it is highly specific in its action against the clostridia, being highly active against *Cl. septicum* and

TABLE I.—*Bacteriostatic Concentration* in vitro *in mg. per* 100 *c.c.*

	Cl. welchii.	*Cl. septicum.*	*Cl. oedematiens.*	*Staph. aureus.*	*Strept. pyogenes.*	Coliforms.	*Ps. pyocyanea.*	*Proteus.*
Flavazole	0·6	0·15	0·3	0·6	0·08	0·6–10	2·5–5	0·3–2·5
5-aminoacridine sulphathiazole	1·25	0·15	0·6	1·25	0·3	0·6	2·5–10	1·25
Proflavine	0·6	0·15	0·15	0·6–1·2	0·15	2·5–10	10–40	1·2–2·5
Suproflavine	0·15	0·08	0·02	0·6	0·01	0·6–2·5	2·5–5	0·6–1·25
Nitracrin	0·15	0·08	0·04	0·6	0·3	2·5	20–40	20–40
V187	12·5	0·8	0·4	250	1·2	400–800	400–800	400–800
V335	1·5	0·8	0·8	40–80	1·2	500–2000	250–2000	500–2000
Marfanil	7·5	3·0	1·5	100–1000	2·5	200	75	1000
Sulphathiazole	200	0·4	200	0·6	0·15–0·6	0·6–40	40	40
Penicillin	0·008	0·008	0·001	0·001	0·0005	—	—	—

only moderately active against the other two. All the other drugs are highly active against all three clostridia and, although most of the drugs are somewhat more active against *Cl. oedematiens*, the bacteriostatic titres of each drug against the three clostridia lie within fairly narrow limits. On the other hand there are wide differences in the bacteriostatic activities of these drugs against the aerobic organisms. Marfanil, V187, V335, sulphathiazole and penicillin are all relatively much less effective against certain aerobic organisms, particularly the Gram-negative bacilli, whereas the acridine compounds show a more uniform and high activity against all the organisms tested. It is also of interest that the acridine-sulphathiazole compounds have lost none of the bacteriostatic activity of their components, and have even greater activity against *Proteus* and *Ps. pyocyanea.*

Toxicity.

With all the substances tested except penicillin an endeavour has been made to maintain a constant relationship between dosage and toxicity by limiting the therapeutic test dose to about $\frac{1}{4}$ to $\frac{1}{3}$ of the maximum tolerated dose by the same injection route. For this purpose mice are injected intramuscularly with increasing quantities of the drug, and the least quantity which causes death is regarded as the maximum tolerated dose. The results of these tests can be judged from the therapeutic doses chosen (Table II). An interesting finding is

that the proflavine-sulphathiazole compound, flavazole, is about 20 times less toxic than proflavine by the intramuscular route whereas, by its constitution, it would be expected to be only half as toxic. Further tests, however, by injection into the peritoneal cavity have shown a much smaller difference in toxicity, so that the discrepancy may be largely ascribed to the much lower solubility of flavazole.

The local toxicity of the drugs has also been examined by the histological method previously described (Selbie and McIntosh, 1943). In the therapeutic doses used, nitracrin, suproflavine and 5-amino-acridine-sulphathiazole are somewhat more toxic than proflavine, whereas flavazole, marfanil, V187 and V335 are about equal to proflavine in toxicity.

Therapeutic Experiments.

Method of testing.

The strains used, *Cl. welchii*, B.S.I. 186 and S 107, *Cl. septicum*, B.S.I. 84, and *Cl. oedematiens*, B.S.I. 193 (Jolly) and B.S.I. 194 (H.I.), and the method of testing the therapeutic efficacy of drugs are the same as those previously described (McIntosh and Selbie, 1943). In each therapeutic experiment 4 to 6 groups of mice are injected intramuscularly in the thigh with 0·2 c.c. of washed organisms suspended in a 2·5 per cent solution of calcium chloride, the infective dose being sufficient to kill all untreated mice within 18 hours and usually about 100 minimal lethal doses. One group of mice is left untreated and the remaining groups are treated 1 hour later each with a different drug, which is injected in solution or suspension in quantities of 0·1 or 0·2 c.c. at the site of the infective inoculation.

The daily incidence of deaths is then noted, and at the end of 3 days, after which deaths are few, an estimate of the efficacy of the drugs in postponing or preventing death is made on the basis of the average number of days survived per mouse over the 3-day period. This figure is obtained by adding up the number of whole days survived by the mice and dividing by the number of mice in the group (Whitby, 1937). Thus in an experiment comprising 6 mice, if 2 mice are dead at 1 day, 1 at 2 days, and 1 at 3 days, leaving 2 survivors, the number of whole days survived is $1 \times 1 + 1 \times 2 + 2 \times 3$, giving a total of 9 for 6 mice and an average of 1·5 per mouse. A more convenient method is to express this figure as the percentage of total survival over 3 days, so that the result of this particular experiment is expressed as 50 per cent ($9/18 \times 100$) of 3 days survived. This method of estimation is a more accurate measure of therapeutic efficiency than the crude survival rate at 3 days, since it allows for the delay in death to the second or third day of treated mice that failed to survive the full 3-day period of observation, and it is of particular relevance here because all the untreated mice died within 24 hours.

Results.

The results of our therapeutic tests against the three chief causal organisms of gas gangrene are shown in Fig. 1 and Tables II and III. These experiments have included parallel tests on penicillin, proflavine and sulphathiazole for purposes of comparison. As will be seen from the results, the activities of these substances against the three anaerobic infections have been comparable with

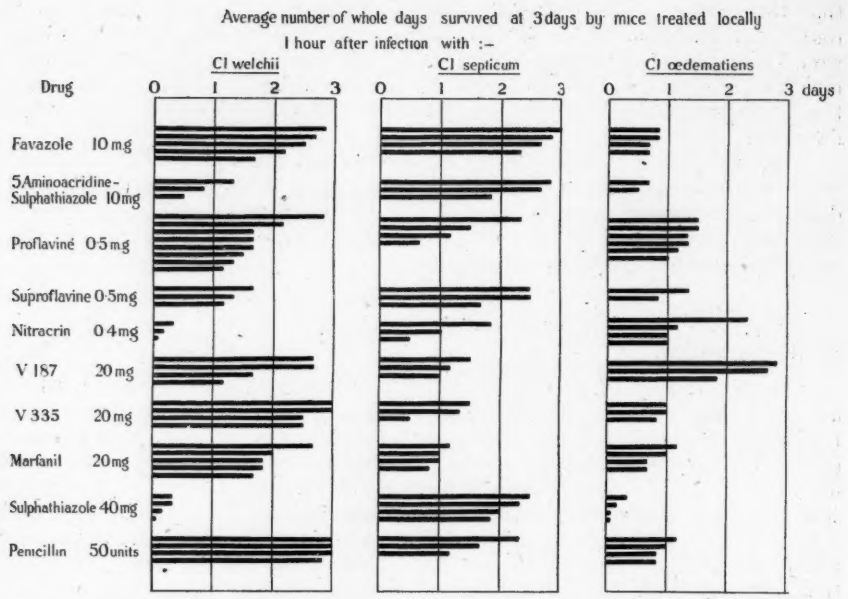

FIG. 1.—Each horizontal line represents one therapeutic test in which 6 mice were treated locally 1 hour after infection; the length of the line indicates the average number of days survived per mouse at the end of 3 days. (For "Favazole" read "Flavazole.")

those obtained previously (McIntosh and Selbie, 1943), so that the conditions of infection and response to therapy do not appear to have altered in any material respect.

The individual tests, each comprising 6 mice, are shown in Fig. 1, where the results are expressed as the average number of whole days survived by the mice at the end of 3 days. It has already been mentioned that the several tests for each drug were made in separate experiments, so that Fig. 1 shows the degree of variation in the response to treatment from one experiment to another. Although this variation has been considerable in a few instances, the results as a whole would appear to be sufficiently uniform to warrant the further analysis presented in Tables II and III.

It is evident from Tables II and III that the three clostridial infections vary greatly in their sensitivity to chemotherapeutic treatment. In the first place, *Cl. oedematiens* infection is in general much less responsive to treatment than the two other infections, in spite of the fact that this organism is at least as sensitive to the direct action of all the drugs used except sulphathiazole (Table I). In the second place, as shown in Table III, the order of efficacy of the drugs varies greatly from one organism to another. Thus, the most active drugs against *Cl. welchii* infection are penicillin and V335, against *Cl. septicum* flavazole, and against *Cl. oedematiens* V187. If, on the other hand, the average efficacy of drugs on all three anaerobes is considered, the most effective are flavazole,

TABLE II.—*Effect of Local Treatment* 1 *Hour after Infection with* Cl. welchii, Cl. septicum *and* Cl. oedematiens.

Drug.	*Clostridium.*	Number of mice.	Survivors at 3 days. No.	Survivors at 3 days. %	Average number of days survived.	Per cent of 3 days survived.
Flavazole 10 mg.	*welchii*	30	23	77	2·4	79
	septicum	24	20	83	2·7	90
	oedematiens	24	0	0	0·8	25
5-Aminoacridine-sulphathiazole 10 mg.	*welchii*	18	2	11	0·9	30
	septicum	18	13	72	2·4	81
	oedematiens	12	0	0	0·6	19
Proflavine 0·5 mg.	*welchii*	48	21	44	1·8	58
	septicum	24	2	8	1·4	47
	oedematiens	36	3	8	1·3	44
Suproflavine 0·5 mg.	*welchii*	18	4	22	1·4	46
	septicum	18	7	39	2·2	74
	oedematiens	12	0	0	1·1	36
Nitracrin 0·4 mg.	*welchii*	18	0	0	0·2	6
	septicum	18	4	22	1·1	37
	oedematiens	24	4	17	1·4	46
V187 20 mg.	*welchii*	24	14	58	2·0	68
	septicum	18	0	0	1·2	41
	oedematiens	18	11	61	2·4	81
V335 20 mg.	*welchii*	24	21	88	2·8	92
	septicum	18	0	0	1·1	37
	oedematiens	18	0	0	0·9	31
Marfanil 20 mg.	*welchii*	30	15	50	2·0	68
	septicum	24	0	0	1·0	33
	oedematiens	24	0	0	0·9	29
Sulphathiazole 40 mg.	*welchii*	24	0	0	0·2	7
	septicum	24	11	46	2·2	72
	oedematiens	24	0	0	0·1	4
Penicillin 50 units	*welchii*	24	23	96	3·0	99
	septicum	18	5	28	1·7	57
	oedematiens	24	0	0	1·0	32
Nil (control mice)	*welchii*	90	0	0	0	0
	septicum	72	0	0	0	0
	oedematiens	60	0	0	0	0

penicillin and V187 with averages of 63 to 65 per cent, the next are V335, suproflavine and 5-aminoacridine-sulphathiazole with averages of 50 to 53 per cent, and the least efficacious are marfanil, nitracrin and sulphathiazole with averages of 28 to 43 per cent.

Some interesting relationships are found when the therapeutic values of the individual drugs are considered. Flavazole is of greater value than either of its components, proflavine and sulphathiazole, against *Cl. welchii* and *Cl. septicum* infections, whereas it is only of intermediate value against *Cl. oedematiens*. 5-Aminoacridine-sulphathiazole is similar to flavazole in its activity against *Cl. septicum* and *Cl. oedematiens*, but is much less active against *Cl. welchii*. Proflavine, as has been noted before (McIntosh and Selbie, 1943), is exceptional

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TABLE III.—*Comparative Efficacy of Drugs Against Anaerobic Infections Expressed as Percentage of 3 Days Survived by Treated Mice.*

Per cent of 3 days survived.	*Cl. welchii.*	*Cl. septicum.*	*Cl. oedematiens.*
100	Penicillin (99) V335 (92)	Flavazole (90) 5-Aminoacridine-sulphathiazole (81)	V187 (81)
80	Flavazole (79) V187 (68) Marfanil (68)	Suproflavine (74) Sulphathiazole (72)	
60	Proflavine (58) Suproflavine (46)	Penicillin (57) Proflavine (47) V187 (41)	Nitracrin (46) Proflavine (44)
40	5-Aminoacridine-sulphathiazole (30)	Nitracrin (37) V335 (37) Marfanil (33)	Suproflavine (36) Penicillin (32) V335 (31) Marfanil (29) Flavazole (25)
20	Sulphathiazole (7) Nitracrin (6)		5-Aminoacridine-sulphathiazole (19) Sulphathiazole (4)
0			

in that it has a moderately high activity against all three types of infections. Of the other acridines tested in this series suproflavine is similar to proflavine except that it is somewhat more active against *Cl. septicum*, while nitracrin is inferior against *Cl. welchii* and *Cl. septicum*, perhaps because of its low solubility.

The results obtained with marfanil, V187 and V335 are of interest in that they are closely related compounds, and would therefore be expected to have similar chemotherapeutic properties. This expectation is upheld in the almost equal activity of marfanil and V187 against *Cl. welchii*, of marfanil and V335 against *Cl. oedematiens*, and of all three drugs against *Cl. septicum* infection. On the other hand V335 and V187 are much more active against *Cl. welchii* and *Cl. oedematiens* infections respectively.

The varying sensitivity of these infections to drugs is also shown in the parallel tests with penicillin and sulphathiazole. Penicillin, in the dosage used, is highly effective against *Cl. welchii* and less effective against *Cl. septicum* and *Cl. oedematiens*, while sulphathiazole is highly active against *Cl. septicum*, and is of little or no value against the two other clostridia.

DISCUSSION.

A comparison of the relative bacteriostatic titres against *Cl. welchii*, *Cl. septicum* and *Cl. oedematiens* of the various drugs used in these experiments (Table I) and the relative efficacy of these drugs against the corresponding infections in mice (Table III) shows that bacteriostatic activity does not necessarily bear a close relationship to chemotherapeutic activity. A high specificity in the in-vitro tests is shown only by sulphathiazole in that it is highly active against *Cl. septicum* and only moderately active against the two other clostridia, and this specificity of action is also shown in the therapeutic tests. With the other drugs of this series the bacteriostatic tests show an almost equal activity for each drug against all three clostridia, whereas the chemotherapeutic tests show great differences in their therapeutic efficiency for the three corresponding infections with the exception of proflavine.

The wide differences that have been found here in the chemotherapeutic activity of drugs against experimental gas gangrene, and in particular, the varying order of efficacy of the drugs from one clostridium to another, may be due to differences in the metabolism of these organisms which, although they have much in common, are recognized as having distinct biological properties. If this is true it would then appear that these differences in metabolism have more decisive effects in determining drug sensitivity in the treatment of infections than in bacteriostatic tests where, as has been shown here, only sulphathiazole of a number of drugs had any specificity in its action against these three clostridia. In therapeutic experiments, however, other factors come into play in determining the effect of chemotherapy, such as invasiveness and toxin production, and these may largely account for the relative insensitivity of *Cl. oedematiens* infection to drug action.

As it is evident, from the results presented here and previously, that no particular drug does at present or is likely in the future to prove a panacea for clostridial infections, we are still convinced of the necessity of using a mixture of drugs for the prophylaxis of clostridial infections in wounds. Such a mixture should be capable of dealing with all the organisms, both aerobic and anaerobic, likely to be met with in wounds, including the Gram-negative bacilli which may promote conditions suitable for the onset of gas gangrene infection.

For this purpose we originally selected a mixture of an acridine (1 per cent proflavine) and sulphathiazole as the most potent and readily available drugs. More recently after clinical trial we suggested that the proflavine in the mixture might be replaced by the new acridine flavazole (2 per cent), which is somewhat less toxic than proflavine and more active against *Proteus* and *Ps. pyocyanea*. This mixture can be enhanced in its activity against *Staphylococcus aureus* and other organisms by the addition of penicillin (McIntosh, Robinson, Selbie, Reidy, Blake and Guttmann, 1945).

SUMMARY.

A number of substances have been tested for their therapeutic activity against *Cl. welchii*, *Cl. septicum* and *Cl. oedematiens* infections in mice.

The results, summarized in Table III, show that these infections differ greatly in their sensitivity to chemotherapeutic substances, and for this reason no one drug can be regarded as an adequate prophylactic against these three infections. An efficient wound antiseptic must therefore consist of a mixture of drugs.

Against *Cl. welchii* infection the most active of the chemotherapeutic substances tested are penicillin and V335 ; against *Cl. septicum* flavazole (proflavine-sulphathiazole compound), and against *Cl. oedematiens* infection the sulphone V187.

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TUMOURS PRODUCED BY 2-ANTHRAMINE.

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THE three aromatic amines, 2-naphthylamine, 2-anthramine and 2-aminofluorene, have been found to induce distant tumours. Hueper and Wolfe (1937) and Bonser (1943) obtained neoplasms of the bladder by feeding 2-naphthylamine to dogs, Shear (1938) hepatomas in mice after injection of 2-anthramine, and Bielschowsky (1944) liver tumours by painting the skin of rats with a solution of 2-aminofluorene in acetone. In this connection some compounds chemically related to 2-naphthylamine may be mentioned. Hueper (1942) discovered the carcinogenic activity of 2-amino-1-naphthol, and Cook, Hewett, Kennaway and Kennaway (1940) found that 2 : 2′-azo-naphthalene and 2 : 2′-diamino-1 : 1′-dinaphthyl produce liver tumours in mice. The last two compounds can be considered intermediaries between 2-naphthylamine and 3 : 4 : 5 : 6-dibenzcarbazole, the carcinogenic activity of which was discovered by Boyland and

Brues (1937). This last substance is the only one of the above-mentioned compounds which has been reported to induce tumours at the site of application as well as acting on the liver.

The carcinogenic aromatic amines are substances of relative simple structure, and therefore specially suited for a study of carcinogenesis. The relationship between chemical structure and biological activity is fairly close, and for these reasons an investigation of the action of 2-anthramine on two strains of rats with known response to 2-acetyl-aminofluorene seemed of interest.

This paper describes the effects produced by painting the skin of rats with 2-anthramine. Dr. Shear has kindly given his permission to quote the results of his experiments with 2-anthramine, of which only a short abstract (1938) has been available. They will be given as stated in Dr. Shear's letter, and discussed together with my results. A short account will be given of essentially negative feeding experiments with 2-anthramine, N-acetyl-2-anthramine and N-acetyl-9-anthramine, and of an experiment in which rats and mice were painted with 9-anthramine.

METHODS.

2-Anthramine (m.p. 238°) was prepared by reduction of 2-amino-anthraquinone according to the method of Ruggli and Henzi (1930). Solutions of this substance in acetone turn from bright yellow to brown in the course of 2–3 weeks. In the first experiment the same solution was used, despite its discoloration. In the other two experiments a batch of 2-anthramine, recrystallized three times from toluene, was used. The solutions were protected from light and made up freshly, whenever the slightest change of colour was observed.

9-Anthramine (m.p. 145–150°) was obtained by nitration of anthracene (Dimroth, 1901) and reduction of 9-nitro-anthracene according to the method of Kaufler and Suchannek (1907). The corresponding acetyl-compounds were prepared by the usual methods. N-acetyl-2-anthramine had the m.p. of 240° and the N-acetyl-9-anthramine of 273°.

The following doses of 2-anthramine were found to be satisfactory, and were employed in all painting experiments:

–10	weeks.	2	drops	(1 per cent	in acetone).	2 ×	weekly.
11–16	,,	3	,,	,,	,,	3 ×	,,
17–22	,,	4	,,	,,	,,	3 ×	,,
23–32 (39)	,,	5	,,	,,	,,	3 ×	,,

19 male rats, 10 albinos of our inbred Wistar strain and 9 piebald rats, were used. The animals were about 6 weeks old at the beginning of the experiment, except for 2 rats, Nos. 8 and 9 in Exp. 3, which were 2 months older.

RESULTS.

Toxicity of 2-anthramine: 2-anthramine is fairly toxic to mice and rats independently of the mode of application. When 4 drops of a saturated solution of 2-anthramine in acetone were applied on to the intrascapular region of the skin of 5 young albino rats, 3 times a week, all animals succumbed in less than 7

weeks. The mortality was low among the albino rats which received increasing doses of 2-anthramine according to the scheme mentioned above. Only one out of 10 animals died on the 222nd day in consequence of an atrophy of the liver. Among the piebald rats, which received the carcinogen for 7 further weeks, 6 out of 9 rats had to be killed because they were very ill or dying. Fortunately only two died before neoplastic changes had become manifest.

No signs of local irritation were observed during the first 23 weeks. 48 hours before the start of the experiment the hair of the intrascapular region was epilated with barium sulphide. Regeneration of the hair occurred at a normal speed, and no loss of hair was observed up to the time when the first tumour was discovered. In the second and third experiment the epilation was repeated in the 23rd week in order to facilitate the recognition of early neoplastic changes. At this stage the regeneration of the fur in the albinos was slow and incomplete; patches were left where only a few isolated hairs reappeared. In the piebalds this disturbance occurred around the 30th week.

The results obtained in groups 1 and 2 are summarized in Table I.

TABLE I.—*Albino Rats, 32 Weeks' Painting.*

Rat No.	Tumour first seen after—	Type of skin tumour.
Group 1 (1944):		
4	180 days	Spindle cell tumour.
2	195 ,,	,, ,,
3	..	Died on 222nd day without tumour.
5	258 ,,	Spindle cell tumour.
1	258 ,,	,, ,, 1 papilloma, 1 basal cell carcinoma.
Group 2 (1945):		
3	196 days	Spindle cell tumour.
4	196 ,,	2 Basal cell carcinomata, 1 papilloma.
2	224 ,,	Spindle cell tumour.
1	238 ,,	,, ,, 1 papilloma.
5	260 ,,	1 Basal cell carcinoma, 1 papilloma.

The spindle cell tumours: the majority of neoplasms observed in groups 1 and 2 were fairly rapidly growing tumours, which were seen first as tiny pinkish nodules, situated below an apparently normal or slightly scaly epidermis. Later they ulcerated the skin and were then frequently covered by sanguineous exudate. They were never seen to regress. Histological study revealed the prevailing type of cell as spindle cells with giant cells scattered throughout the tumour tissue. In some cases these mono- and polynuclear giant cells were very numerous in the uppermost layer of the corium just below the epidermis. The epidermis showed varying degrees of hyperplasia where it was still intact, reaching considerable dimensions in the periphery of the larger spindle cell tumours. Only slight hyperplasia was seen when earlier stages of these neoplasms were studied. Sebaceous glands and hair-follicles which remained well preserved in the painted area were destroyed by the growing spindle cell tumours, and so were the elastic and collagen fibres of the corium. The tumours broke through the panniculus carnosus early (Fig. 1 and 2). Silver staining (Foot, Robb Smith) revealed

the presence of an argentophile reticulum (Fig. 3). Two of these tumours were transplanted into young animals of the same strain. The first graft grew slowly, but the second generation could be retransplanted as early as the third week. No change in the histological structure could be observed after three generations of transplants (Fig. 4 and 5). In the second group of rats an attempt was made to obtain information on the origin of the spindle cell tumours. Even at a time when these neoplasms had only a diameter of 2–3 mm., however, the spindle cells were always in close contact with the overlying epidermis. In some sections the greatest density of malignant cells was found in the corium midway between panniculus carnosus and epidermis, but this was not the rule. Inflammatory changes were inconspicuous, as long as there was no ulceration.

Basal cell carcinoma: 5 neoplasms of this type have been found, two of them in the same rat. Their structure was very similar to basal cell carcinoma of the human skin. Like these they grew very slowly, and remained for a long time confined to the superficial layer of the corium. In stained sections they seemed to lie in spaces well separated from the more or less hyperplastic epidermis, which remained intact up to the time when the animals were sacrificed. Early ulceration, as in rodent ulcer of the human skin, has not been seen in 2-anthramine painted rats, but was observed twice in rats which had received a diet containing 2-acetyl-aminofluorene. The blood supply of these tumours was poor, and in consequence degeneration in the centre, followed by formation of cysts, occurred at an early stage. The cells composing these tumours had a scanty cytoplasm; their shape varied from polyhedral to spindle. The former had a vesicular, the latter a more compact and hyperchromatic nucleus (Fig. 6–8).

Besides the two types of neoplasm described above, a few papillomas, most of them still benign (Fig. 6), or just in the earliest stage of malignancy (Fig. 9), were seen. These had the same structure as the well-known early skin tumours produced in mice by benzpyrene or tar.

The results obtained in group 3 are summarized in Table II.

TABLE II.—*Piebald Rats, 39 Weeks' Painting.*

Rat No.	Tumour first seen after—	Type of skin tumour.	Distant tumour.
3*	..	Died on 119th day without tumour.	..
1*	224 days	1 Papilloma	..
7*	247 ,,	2 Papillomas	Tumour of duct. acoust. ext.
2*	273 ,,	1 Papilloma	..
6	280 ,,	Squamous carcinoma, basal cell carcinoma	Tumour of duct. acoust. ext.
4*	281 ,,	2 Papillomas	..
9	282 ,,	Spindle cell tumour, 2 papillomas	..
5	293 ,,	2 Spindle cell tumours	Adenocarcinoma of caecum.
8*	..	Died on the 311th day without tumour.	..

* These animals had to be killed because they were seriously ill.

In the strain of piebald rats 2-anthramine produced tumours of the skin at a later date, and only 2 out of the 9 animals developed rapidly growing spindle cell tumours. Six animals had to be killed because they were seriously ill, and 3 of these had minute papillomatous growths. Small invasive anaplastic foci arising from the hyperplastic epidermis were already present in rats 7 and 2, but the process was not far enough advanced to classify these neoplasms as squamous carcinomata. Retention cysts were frequently seen in the neighbourhood of the papillomatas. Apart from these definitely neoplastic processes there were pathological changes in the subepidermal tissue, which were more difficult to assess. Their main characteristics were: an accumulation of spindle cells and round cells, and in rat 9 chondroid tissue was found below the hyperplastic down-growing epithelium (Fig. 10). It consisted of separate vesicular cells with a compact nucleus, surrounded by collagenous fibres forming its interstitial substance.

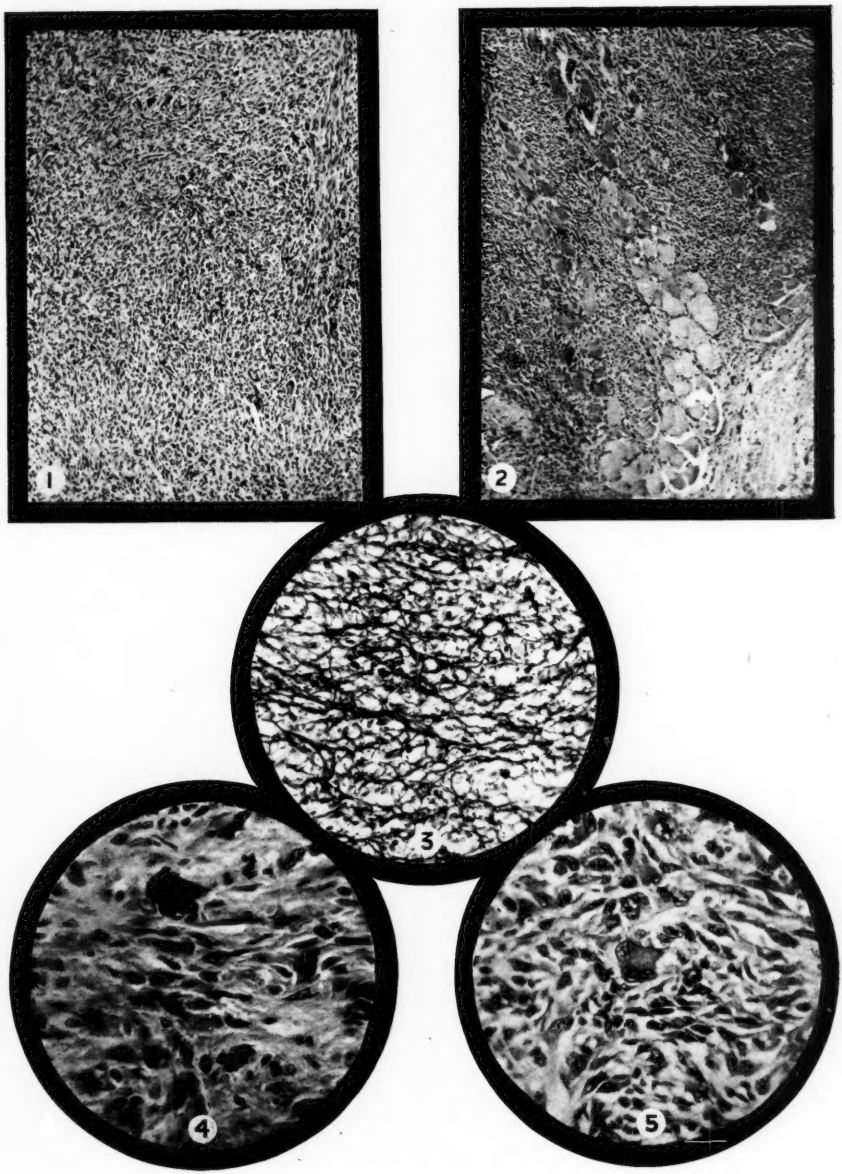
Three distant tumours were found in this group, 1 carcinoma of the caecum and 2 squamous, keratinizing carcinomata of the ductus acousticus externus. The former tumour, an adenocarcinoma, producing abundant mucin and invading the muscularis, had some features which have not been observed in tumours of the intestine induced by 2-acetyl-aminofluorene, but the other 2 neoplasms were indistinguishable from the many tumours of this kind seen in rats which had received 2-acetyl-aminofluorene by mouth.

Dr. Shear's Experiments.

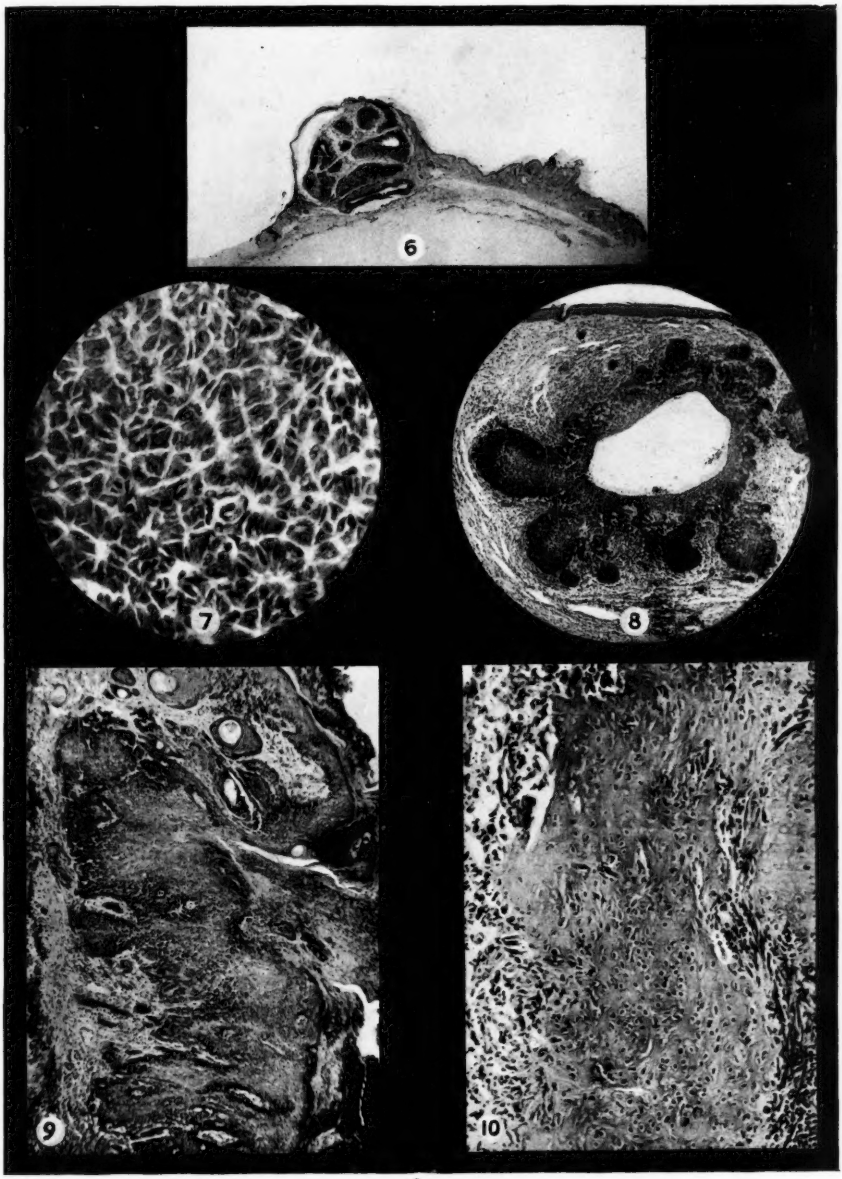
Experiment 1: Solid—5 mg. solid compound (m.p. 234–236°) were injected subcutaneously into 12 mice. The injection was repeated once. The mice lost weight over a period of three months and went downhill, obviously because of the toxicity of the compound given in this way.

Experiment 2: Solution—0·25 c.c. of a saturated solution of 2-anthramine in lard filtrate was injected subcutaneously in each of 20 albino mice. Half the mice were strain A males; the other 10 were "market" albino females. The experiment lasted from 16 to 20 months. Significant changes in the liver were found in 8 out of the 10 heterozygous albino mice. One showed multilocular cysts. Two livers showed cirrhotic changes. Multiple hepatomas occurred in 6 mice. One mouse showed primary liver carcinoma. One of the hyterozygous mice showed no pathological changes but did have lymphosarcoma, which may have been spontaneous. From 7–10 injections were given.

FIG. 1.—× 68. Spindle cell tumour. (Group 2, rat 3.)
FIG. 2.—× 71. Spindle cell tumour. (Group 3, rat 5.)
FIG. 3.—× 250. Silver staining. Detail from tumour shown in Fig. 2 (reticulum).
FIG. 4.—× 300. Detail from tumour shown in Fig. 1.
FIG. 5.—× 300. Third generation of transplant, derived from tumour, Fig. 1.
FIG. 6.—× 6. Left: Basal cell carcinoma. Right: small papilloma.
FIG. 7.—× 300. Detail from basal cell carcinoma shown in Fig. 6.
FIG. 8.—× 23. Basal cell carcinoma. (Group 2, rat 4.)
FIG. 9.—× 54. Early squamous carcinoma. (Group 3, rat 6.)
FIG. 10.—× 110. Chondroid tissue. (Group 3, rat 9.)



Bielschowsky.



Bielschowsky.

Feeding experiments with 2-anthramine (Exp. 4–6) and N-acetyl-2-anthramine (Exp. 7–9).

Exp. 4 : 5 young albino rats received 6 mg. of 2-anthramine daily for 5 days. Two days later all were dead.

Exp. 5 : A group of 5 male albino rats received 2 mg. for 1 week, 1 mg. for 2 weeks, 2 mg. for 3 weeks. All died between the 42nd and 44th day.

Exp. 6 : 5 rats received 1 mg. for 21 weeks ; no tumours were found.

Exp. 7 : 5 rats received 2 mg. of N-acetyl-2-anthramine for 2 weeks, 3 mg. for 1 week and 4 mg. for 2 weeks daily. All died in the sixth week with ascites and hydrothorax.

Exp. 8 : 5 male rats received daily for 9 weeks 2 mg. of the same compound. Three died in the 11th week ; the 2 survivors received 2 mg. daily for 7 more weeks, after an interval of 8 weeks. The livers of both rats were cirrhotic. Hydrothorax and ascites were present.

Exp. 9 : 10 rats received 2 mg. of the acetylcompound for 7 weeks, 1 mg. in the 8th week, 2 mg. in the 9th and 10th week, and 1 mg. during the following 4 weeks.

One animal died in the 14th week. The drug was omitted for 5 weeks and the survivors received 1 mg. for 6 further weeks. The last animal died in the 39th week. No tumours were found ; some of the livers showed cirrhotic changes, fatty degeneration and a few isolated cysts. The kidneys showed degeneration of the tubular epithelium, many casts and cystic dilatation of the tubules.

9-Anthramine and N-acetyl-9-anthramine.

Exp. 1 : 5 male albino rats and 5 albino mice were painted with 9-anthramine for 40 weeks. No tumours appeared up to the time of writing (54 weeks).

Exp. 2 : 5 male piebald rats received daily 6 mg. of N-acetyl-9-anthramine for 36 weeks. The animals grew at a normal rate and there was no death within one year.

DISCUSSION.

Tumours of the skin have been obtained with relative ease in three groups of rats, by painting with 2-anthramine. This result was unexpected. Shear (1938) had obtained liver tumours in mice, and it seemed therefore more likely that 2-anthramine, like 2-aminofluorene, would produce this type of tumour when applied to the skin of rats. But although a few distant neoplasms appeared in the piebald strain, cancer of the liver has not been observed so far. This might be due to an exclusive use of male rats. Shear obtained his positive results in female mice. Furthermore, the epidermis of rats has been considered to be fairly resistant to the action of most chemical carcinogens. Whereas subcutaneous injections of carcinogenic hydrocarbons induce as many sarcomata in rats as in mice, painting of the skin with tar or with one of the very potent hydrocarbons like methylcholanthrene is only rarely followed by development of neoplasms (Bachmann *et al.*, 1937). The only exception so far seems to be 9,10-dimethyl-1,2-benz-anthracene (Berenblum, 1945). It has been suggested (Glücksmann, 1945) that there might exist a correlation between susceptibility to a carcinogen and thickness of epidermal layering and size of hairs and density of haircoat. However, the results of Hueper (1945), who studied the action of methylcholanthrene in hairy and hairless rats and found the haired even more refractory than the hairless strain, cause one to doubt whether such variations in

anatomical structure account for the different susceptibility of the epidermis of mice and rats to the action of tar and most of the carcinogenic hydrocarbons. There is no *a priori* reason why there should not be a similar degree of species specificity in susceptibility to carcinogens as to other drugs. To quote a recent example, wild Norway rats are very susceptible to α-naphthyl-thiourea, whereas Alexandrine rats are fairly resistant (Richter, 1945). It seems possible that the skin of rats is more sensitive to the action of 2-anthramine than is that of mice and rabbits. When terms like susceptibility or resistance are used in cancer research, it is important to indicate species, organ and compound.

Tumours composed of three different types of cells have been observed—squamous epithelium, basal cell epithelium and spindle cells. The first group need not be discussed, since neoplasms of this kind have been described many times. Basal cell tumours seem to be very rare in rodents. Besides the five neoplasms which were found in 19 rats painted with 2-anthramine, 3 basal cell carcinomata have been seen in a group of 300 albino rats, given a diet containing 2-acetyl-aminofluorene. This proves that this type of tumour can be produced by more than one compound, and by different routes of administration.

The nature of the spindle-cell tumours has not been established as yet. Similar neoplasms have been found in mice after tarring, and 20 years ago these were the subject of much discussion. The question whether the spindle-cell tumours induced by tarring the skin of mice were sarcomata or carcinomata was never settled. Whereas Roussy and Leroux (1922) insisted that this type of neoplasms always originated from epithelial cell elements, Fibiger (1923) believed that at least some of them were true sarcomata. Recently it has been shown (Bradbury, Bachmann and Lewisohn, 1941) that some very potent carcinogens like 9,10-dimethyl-1,2-benz-anthracene ulcerate the epidermis of mice after only a few applications, with the result that the deeper layers of the skin become exposed to direct contact with the carcinogen. It seems likely that spindle-cell tumours, which arise under such conditions, are sarcomata. But there exists good evidence that tumours of mesenchymal origin can be produced in the skin under conditions where the epidermis is intact. Grady, Blum and Kirby-Smith (1941, 1943) describe many sarcomata, some of which originated probably from muscle cells, a few haemangio-endotheliomata and an osteochondrosarcoma of the ear of mice, exposed to ultraviolet radiation. Their work shows that it is not always the outermost layer of the skin which responds first to the action of a cancer-producing stimulus, although the epidermis receives more of it than the corium. The material presented in this paper does not allow a categoric statement whether the 2-anthramine-induced spindle cell tumours are sarcomata or carcinomata. Although some of them seemed to have all characteristics of a sarcoma and their structure remained unchanged during successive transplantations, in other tumours of this kind groups of cells were found which resembled epithelial rather than mesenchymal cells. Epithelial cells in rodents readily change their appearance under the influence of a carcinogen and become spindle cells. Spontaneous mixed tumours containing malignant epithelium and mesothelium have been described in rats (Dunning, Curtis and Maun, 1945). Ewing (1940) was very doubtful of so-called experimental carcino-sarcomata : he was convinced that at least some of them were anaplastic carcinomata.

The small number of rats employed in the experiments presented in this paper does not warrant the conclusion that distant tumours can be obtained

with 2-anthramine only in the piebald strain. In Shear's experiment only the "market" albino mice developed liver tumours, whereas in A strain males no significant changes occurred. The difference of sex and strain makes the interpretation of the results difficult.

The occurrence of tumours of the ductus acousticus externus in this series is worth noticing. These tumours were first described by Wilson, DeEd and Cox (1941) in rats receiving 2-acetyl-aminofluorene. They occur very frequently in both my strains after treatment with 2-acetyl-aminofluorene (Bielschowsky, 1944). That 2-anthramine can produce the same type of tumour seems to point to a similar underlying mechanism in the pathogenesis of these neoplasms.

SUMMARY.

In two strains of rats benign and malignant tumours of the skin have been produced after painting with 2-anthramine.

Distant tumours developed in one strain.

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SURVEY OF PAPERS.

CARR has estimated the amount of virus that is transferred from a Rous sarcoma to non-filterable tumours growing in the same fowl. Only small amounts of transferred virus can be detected and are attributed to dissemination in the vascular system. There is no evidence that the non-filterable tumours adsorb the Rous virus, or that they contain an antigen capable of producing antibodies to the Rous virus (p. 1).

HARRISON AND FULTON have studied the histological changes in the spleen of golden hamsters infected with *Leishmania donovani* and the effects of treatment with stilbamidine. In untreated animals there is considerable enlargement of the spleen and its Malpighian bodies, while in successfully treated animals the spleen is moderately enlarged for as long as nine weeks after the end of treatment (p. 4).

MEDAWAR finds that epidermal cell-division is brought to a standstill, without immediate cell death, in skin homografts transplanted to rabbits which have been immunized by an earlier grafting of skin from the same donor. The reaction is highly specific towards the skin of the rabbit that provided the immunizing dose of homografts (p. 9).

MEDAWAR finds that leucocytes transplanted from one rabbit to another may establish a typical complete immunity towards skin later transplanted from the same donor. Their immunizing effect is at least 18 times more effective through the intradermal route than through the intravenous. The intradermal injection of erythrocytes does not confer skin immunity, and the grafting of foreign homologous skin does not elicit the formation of donor red cell agglutinins, (p. 15).

MILES found that the sera of convalescent cases of post-arsphenamine hepatitis fixed complement in the presence of liver extracts from fatal cases when the incidence of this condition was high, but that this reaction was negative in later cases when the incidence fell after precautions were taken to prevent transmission by syringes. Attempts to find a specific complement-fixation reaction in infective hepatitis and homologous serum hepatitis failed (p. 25).

CULLUMBINE AND RYDON have demonstrated the presence of leukotaxine in blister fluids and in phosgene lung oedema fluid and have produced it from a number of proteins by enzymic digestion. Examination of a partially purified preparation shows that leucotaxine may be a polypeptide of comparatively low molecular weight, containing tryptophan as an essential component (p. 33).

MCINTOSH AND SELBIE show that *Cl. welchii*, *Cl. septicum* and *Cl. oedematiens* infections in mice differ greatly in their sensitivity to local treatment with penicillin, sulphathiazole, acridines and sulphones. Of the substances tested the most active against *Cl. welchii* are penicillin and the sulphone V335, against *Cl. septicum* flavazole (proflavine-sulphathiazole compound), and against *Cl. oedematiens* infection the sulphone V187 (p. 46).

BIELSCHOWSKY has produced benign and malignant tumours of the skin in two strains of rats by painting the skin with 2-anthramine. In one strain distant tumours were also produced (p. 54).